CADMIUM INTOXICATION AND THE INFLUENCE

OF DIETARY SULFUR CONTAINING AMINO

ACIDS ON CADMIUM TOXICITY

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Dedicated to

MY HUSBAND, Mingyu Ye

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PREFACE

This dissertation is composed of six chapters. Each chapter by itself is a complete and independent manuscript except chapter I and VI. This work is an attempt to partially elucidate some of the biochemical mechanisms of cadmium, an environmental pollutant, induced toxicity, and to characterize the influence of some dietary components on cadmium toxicity. Chapter I is a review of the literature of cadmium sources, absorption, distribution, toxicity, detoxification and interactions with other metals. Chapter II is a description of HPLC-EC (High Performace Liquid Chromatography with Electrochemical Detector) method used in quantitating hydroxyl free radical generation in cadmium induced testicular toxicity which has been published in Journal of Liquid Chromatography (Shen et al. 1995). Chapter III describes cadmium-induced biochemical changes in mouse testicular toxicity. These include changes in Na^+, K^+-ATPase, glutathione, lipid peroxidation and free radical generation. In this chapter, the results of pretreatment with vitamin E on cadmium-induced biochemical changes in mouse testicular toxicity is presented. This has been published in Archives of Environmental Contamination Toxicology (Shen and Sangiah, 1995). The effects of different levels of methionine and cystine in the diet on cadmium-induced biochemical changes ($CdCl_2$ 100ppm in drinking water for 10 weeks) are described in Chapter IV. Chapter V deals with the influence of various levels of methionine and cystine in the diet on cadmium and cadmium-induced changes in copper, iron and zinc distribution in major target organs. The summary and conclusions are presented in Chapter VI.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Cadmium (Cd) is a rare metallic element that occurs naturally in the earth's crust and it is present in almost all types of food. Shellfish, wheat and rice accumulate very high amounts of Cd. All soils and rocks, including coal and mineral fertilizers, contain some Cd. Cd is often found as part of small particles present in air. Occupational and environmental pollution are the main sources of Cd exposure. Cd has a very long biological half-life in the body and acts as a cumulative poison in human and higher animals. Exposure to Cd causes anemia, hypertension, hepatic, renal, pulmonary and cardiovascular disorders. It is considered as a possible mutagen, teratogen and carcinogen.

Physical and Chemical Characteristics

Cd was discovered in 1817 by Strohmeyer and Herman in Germany (Recht 1978). It falls in group IIB of the Periodic Classification between zinc and mercury (Aylett 1979). Cd lies at the end of the second transition series of elements, with a completed 4d shell of electrons. In chemical reactions, up to two electrons may be removed to form cations. The s-p energy separation is less than that for either zinc or mercury, and this suggests that excitation to the valence state may be easier for Cd than for its neighboring elements.

Cd has a relatively high vapor pressure. Its vapor is oxidized rapidly in air to produce Cd

oxide. When reactive gases or vapor, such as carbon dioxide, water vapor, sulfur dioxide, sulfur trioxide or hydrogen chloride are present, Cd vapor reacts to produce Cd carbonate, hydroxide, sulfite, sulfate or chloride, respectively (Friberg et al. 1992).

The most common oxidation state for Cd is Cd(II). It has proved possible to isolate a few compounds which formally contain Cd(I); these are all analogous to the better-known mercury(I) derivatives in that they contain the dimeric cation $(Cd-Cd)^{2+}$ with a metal-metal bond. There is also evidence for extremely short-lived Cd^+ species in solution, formed as intermediates in the reduction of Cd^{2+} to Cd metal. Coordination numbers of Cd(II) provide a revealing approach to the differing types of chemical behavior that can be exhibited. The most common coordination arrangements are tetrahedral and especially octahedral.

Cd shows a marked tendency to form bridged species, both in solids and solutions. In solids, bridging will always have the effect of increasing Cd's coordination number, but in solutions this will not necessarily be the case, because coordinating solvent molecules may be lost in compensation. Another point concerns hydrolysis effects, leading to formation of Cd-OH species; these may be more important in aqueous systems than has been generally recognized.

Sources of Human and Environmental Exposure

The proportion of Cd in the earth's crust is estimated to be between 0.1 and 0.2 ppm. It is found mainly in zinc, lead-zinc and lead-copper-zinc ore. Cd is a byproduct of zinc production. Numerous human activities result in the release of significant quantities of Cd into the environment. The major sources of anthropogenic Cd release can be divided into three categories (Friberg et al. 1992). The first is those activities involved in the mining, production,

and consumption of Cd and other non-ferrous metals. The second category consists of inadvertent sources where the metal is a natural constituent of the material being processed or consumed. Sources associated with the disposal of materials make up the third category.

Cd is released into air, land, and water by human activities. Cd pollution of the environment may result from a number of industrial and agricultural activities. The most important sources are mining, refining and smelting operations utilizing ores rich in Cd (particularly some sulfide ores primarily used in zinc and lead production), and manufacturing of Cd batteries and pigments. Coal may contain traces of Cd which will be emitted during combustion. The soil may be contaminated from the use of Cd-rich sewage sludge or phosphate as fertilizers in agriculture. Once soil has been contaminated, it may remain so far decades and the Cd may be taken up by the crops grown in that soil.

Industrial production and use of this element has been significantly expanded for the past three decades. It is estimated that Cd is being released into the atmosphere at a rate of about 1.5 million kg/yr (Friberg et al. 1992). Tobacco smoke is an important source of human exposure to Cd through inhalation.

Human uptake of Cd occurs via the inhalation of air and the ingestion of food and drinking water. In contaminated areas, Cd exposure via food may be up to several hundred μg/day. In exposed workers, lung absorption of Cd following inhalation of workplace air is the major route of exposure.

Cd Absorption, Transport, Distribution and Excretion

In human, the primary routes of Cd exposure are through the respiratory and gastrointestinal tracts via exposure to Cd-contaminated air, food, or water.

Gastrointestinal(GI) absorption of Cd by mammals is low and is influenced by many factors. Absorption is reported to be 1 to 2% in goats, 5% in swine and lambs, approximately 16% in cattle and 3 to 8% in humans (Ragan and Mast 1990). All studies seem to indicate at least a 2 fold greater absorption from the lungs than from the GI tract. Absorption of parenterally administered Cd is efficient. Whole body measurements have shown that rats retain greater than 90% of either an intravenous or intraperitoneal dose after 30 days (Moore et al. 1973) and mice retain 88% of a single subcutaneous dose after 25 days (Shaikh and Lucis 1972).

Many factors such as the dietary content of other metals, and protein malnutrition influence the absorption and toxicity of Cd. Age also influences the absorption and toxicity of Cd. The absorption of Cd in young mice is higher than in adults (Ragan and Mast 1990). But the ratios of LD_{50}'s in adults to those in newborn animals range from 0.002 to 16 or more indicating that Cd is considerably more toxic in adult animals than in newborns (Samarawickrama 1979). The amount of Cd deposited in the lung compartment depends mainly on the concentration in air and the particle size (Recht 1978). Data from experimental animals and human studies have shown that inhalation of Cd compounds can produce both acute and chronic effects on the respiratory system (Friberg et al. 1992).

After absorption from the intestinal mucosa, the metal is transported in blood to the liver, kidneys and other tissues. It is stored principally in the liver and kidneys where more than half of the body burden is deposited. There is no doubt that after long-term exposure, Cd accumulates mainly in the kidneys (Gross et al. 1976). Highest Cd concentrations are generally found in the renal cortex. The Cd concentrations in most tissues increase with age.

Cd has a very long biologic half-life in the body and it is taken up from the blood into the liver, where incorporation into metallothionein (MT) occurs. MT is an important transport and

storage protein for Cd and other metals. Cd can induce MT synthesis in many organs including the liver and kidney. Cd is then slowly released from the liver into the blood for transport to other organs, especially the kidneys. The ability of humans and animals to eliminate Cd from the body is limited. Small amounts of Cd are lost in urine, hair and sweat. The feces represent the principal excretory route for Cd.

Cd Toxicity

Cd is toxic to virtually every system in the animal body, whether ingested, injected, or inhaled. The toxic effects of exposure to Cd compounds include anemia, dermatitis, testicular degeneration or atrophy, reduced growth rate, liver and kidney damage, cardiovascular disorders, pulmonary edema and emphysema, teratogenic malformations and increased mortality. Cd increases the susceptibility of different animal species to various infectious diseases and bacterial endotoxins. This is related to its immunosuppressive effect (Samarawickrama 1979). Experiments (mammalian cells *in vitro* and animals) showed that Cd, in certain forms, had mutagenic properties. Studies in animals have provided conclusive evidence that injection of Cd causes local sarcoma at the site of injection as well as interstitial tumors of the testis (Heinrich 1988). A study has shown that inhalation of Cd aerosol has strong carcinogenic effects in rat (Elinder and Kjellstrom 1986).

Exposure to high doses of Cd can give rise to morphologic changes in the liver (Friberg 1950; Stowe et al. 1972). After exposure to most Cd compounds, high concentrations of the metal will be found in liver. The overt signs of acute toxicity in the liver which are seen shortly after parenteral administration of Cd are most likely related to nonmetallothionein-bound Cd. When metallothionein has been produced in the liver, it sequesters Cd and thereby decreases

its toxicity. Friberg (1950) demonstrated fibrotic changes in the liver of rabbits exposed to repeated subcutaneous injections of Cd. Cd enhances MT production, serum protein dyscrasias, enhanced serum aspartate transaminase(AST) and alkaline phosphatase levels. Morphological changes of limited severity appear at liver Cd concentration greater than 60 μg/g wet wt. (Faeder et al. 1977).

When rabbits given 2 and 1.25mg Cd/kg (i.v.), respectively, they survived for more than 72 hr. After 24 hr of Cd exposure, there was a 4-fold increase in aspartate aminotransferase (AST) activity, but it reached within normal range again 72 hr later (Andreuzzi and Odescalchi 1958). Dudley et al (1982) performed similar studies in rats. Injections of 3.9 mg Cd/kg (i.v.) brought about severe hepatic damage within hours, including a dramatic increase in plasma enzymes released from the liver, as well as pronounced morphological changes.

Assay of enzymes from liver homogenates provides a method to identfy the early signs of impaired liver function. Rats given Cd in drinking water showed an increase in the activity of phosphorylase and decrease in aldolase activity, indicating that Cd may interfere with carbohydrate metabolism in liver (Sporn et al. 1970). Rats given larger amounts of Cd in food over shorter periods of time exhibited an influence on oxidative phosphorylation in liver mitochondria. Further evidence that long-term Cd exposure may affect carbohydrate metabolism in the rat has been provided by Merali et al. (1974).

Other biochemical studies on the effects of long-term exposure of liver enzymes to Cd are also available. Copius Peereboom-Stegeman et al. (1979) gave rats subcutaneous (s.c.) injections of 0.5 mg CdCl$_2$/kg three times a week. After 12 to 13 weeks, liver homogenates displayed an increase in activity of alkaline phosphatase and a decrease in glycogen content.

The degree of occupational exposure to Cd was found to be correlated with urinary Cd excretion, gross proteinuria, and abnormalities of the urinary protein electrophoretic pattern

suggestive of glomerular damage (Lauwerys et al. 1974). Morphological changes and histological lesions of the renal tubules were found in rats exposed to Cd (Kawai et al. 1976; Itokawa et al. 1978).

It is well documented that exposure to Cd will cause impairment of the kidneys. This is the critical effect after long-term exposure. Cd exposure can induce both acute and chronic effects in the kidneys of animals and humans. The major damage is done to the renal tubules, but at higher doses glomeruli and the renal blood vessels may also be affected. The mechanism of the renal effects is related to Cd bound to metallothionein(MT), which is freely filtered through the renal glomeruli and reabsorbed in the proximal tubules. After entering the lysosomes of proximal tubular cells, the metallothionein is catabolized and "free Cd" is released, which probably interferes with zinc-dependent enzymes and causes damage to the metabolism of the renal tubular cells. Single s.c. injections of Cd to rats resulted in renal tubular lesions (Favino and Nazari 1967). Two s.c. injections of Cd produced renal lesions in rabbits (Foster and Cameron 1963).

The first study of proteinuria was reported by Friberg in 1950. Axelsson and Piscator (1966) gave rabbits daily s.c. injections of Cd 0.25 mg/kg, 5 days/week. After 23 weeks, there was considerable proteinuria and excretion of Cd. The Cd induced proteinuria is the most characteristic sign of Cd-induced renal tubular damage. It involves an increase of all plasma proteins in urine and particularly an increase of low molecular weight proteins. The proteinuria is mainly due to a renal tubular reabsorption deficiency, which similarly affects the reabsorption of all proteins, glucose, amino acids and phosphate from the glomerular filtrate. Cd-induced proteinuria in rats has also been shown after long-term oral exposure (Bernard et al. 1976). The increase of different proteins, glucose, and amino acids in urine caused by Cd may be interpreted as indicators of decreased renal function. Increased urinary excretion

of the enzymes alkaline phosphatase, AST, ALT, and LDH was found at the same time as proteinuria (after about 10 weeks) in rabbits which were treated with Cd (0.5 mg/kg, s.c.) for 21 weeks (Nomiyama 1982).

Testes are one of the most sensitive tissues to the acute toxic and chronic carcinogenic effects of Cd (Waalkes and Oberdoster 1990). The most prominent acute effect is testicular necrosis. Hemorrhage, edema and necrosis with destruction of seminiferous tubules has been reported in previous studies (Gunn et al. 1968). Acute testicular necrosis may be induced in several animal species after single parenteral administration of Cd. There is a very rapid progression of changes in the testis and the proximal end of the caput epididymis. The testis first became swollen and dark red or purple. Weight then decreased rapidly and the testis became small, hard, and yellowish. The testicular lesion is due primarily to vascular damage. Cd gives rise to increased permeability of the testicular capillary blood system. Capillary damage gives rise to massive vascular escape of fluids and blood substances into the interstitium which subsequently causes edema and circulatory stasis. Further, ischemic necrosis of the testis due to Cd mimics the degenerative changes which result from permanent occlusion of the testicular blood supply (Koizumi et al. 1992). Cd interacts with structural and functional components of cell membranes and inhibits ATPase and cellular transport of Na^+, and Ca^{2+} by interaction with functional sulfhydryl groups of the membrane bound Na^+,K^+- and Ca^{2+}, Mg^{2+}-ATPases. A previous study has demonstrated that Cd inhibited ATPase enzymes system in the brain, liver and kidney of rat (Rajanwa et al. 1981). No studies have been reported on the effects of Cd on testicular Na^+,K^+-ATPase *in vitro* and *in vivo*.

ATPase, an integral part of the cell membrane plays an important role in the active transport of Na^+ and K^+ across cell membrane. It is the primary driving force for other membrane associated transport systems or channels (Ca^{2+} channels, Na^+/Ca^{2+} and Na^+/H^+

antiporters and Na$^+$-dependent transporters for phosphate, glucose and amino acids). Thus, Na$^+$, K$^+$-ATPase plays a vital role in a major communication system linking the extracellular signals to the intracellular medium, not only of neural tissues but also of non-neural tissues. The inhibition of these vital enzymes by cadmium could be damaging to the testicular cells leading to an earlier stage of edema followed by later stage of degeneration and necrosis. However, although most of the toxic metals including Cd are known to bind with sulfhydryl groups of both α and β-subunits of Na$^+$, K$^+$-ATPase, the exact manner by which the Cd brings about the inhibitory effects on Na$^+$, K$^+$-ATPase varies from species to species and perhaps also from organ to organ (Kinne-Saffran et al. 1993).

Cd also inhibits DNA-dependent RNA-polymerase activity and affects nucleic acid synthesis and function. Cd increases production of respiratory CO_2, causes hyperglycemia, glycosuria, increases phosphorylase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities, and decreases hepatic aldolase activity(Aylett 1979).

Detoxification

Glutathione (GSH)

GSH is a nonprotein thiol which is involved in cellular protective mechanisms that modulate responses to toxic agents. GSH was isolated and named by the English biochemist Fredrick Gowland Hopkins in 1921. The structure of GSH was established as r-glutamylcysteinylglycine (r-Glu-Cys-Gly) through chemical analysis, acid-base titration, degradation, and synthesis (Kosower 1978). GSH is assembled from its constituent amino acids in two separate reactions. The first step involves formation of a L-glutamyl linkage between L-glutamate and L-cysteine, catalyzed by r-glutamyl-cysteine synthetase. In the

second step, GSH synthetase catalyzes the addition of glycine to r-glutamyl-cysteine to form GSH in the reduced state (Reed and Beatty 1980). Probably the most important source of cysteine is dietary intake, either directly as a constituent of dietary protein or indirectly as methionine. Methionine is capable of providing cysteine equivalents for incorporation into GSH. GSH is synthesized intracellularly and a major fraction of the transpeptidase is on the external surface of the cell membranes. GSH transported across cell membranes interacts with r-glutamyl transpeptidase. Under normal conditions, much of the GSH in cells is present as free GSH. Oxidized glutathione(GSSG) is usually present in cells in much smaller concentrations than those of GSH. Intracellular GSH is converted to GSSG by selenium-containing GSH peroxidase, which catalyzes the reduction of H_2O_2 and other peroxides (Meister and Anderson 1983).

The structural integrity of the cell membrane and the membranes of intracellular organelles depend upon an appropriate GSH status. The primary effects involve regulation of the thiol/disulfide ratio in proteins through interchange reactions, and protection of lipids against peroxidation through the interception of free radicals and the decomposition of lipid hydroperoxides. The membrane is damaged by chemical challenges in the absence of cellular GSH. Various membrane functions, including ion and sugar transport, mitochondrial functions, the release of neurotransmitters, and the action of hormones, are apparently dependent upon a suitable thiol-disulfide balance within the proteins involved, a balance which can be altered by a change in the GSH status of the cell or system (Kosower 1978). Probably the most widely known biological role of GSH is that of conjugation with foreign compounds or their metabolites. This role fulfills the dual function of enhancing excretion and detoxifying reactive chemical species. Conjugation with GSH not only facilitates the excretion of foreign compounds or their metabolites, but serves as well to intercept highly reactive electrophilic

compounds before they can covalently bind to tissue nucleophiles leading to toxic and/or mutagenic sequelae (Reed and Beatty 1980). Conjugation with GSH has always been considered to decrease the reactivity of a compound, resulting in decreased toxicity. The activities of many enzymes are influenced by GSH and such effects may reflect significant physiological regulatory phenomena. Many observations show that the radiosensitivity of cells depends on the intracellular thiol level. The role of GSH in the prevention of oxygen toxicity is closely related to phenomena associated with radiation. Increased oxygen tension leads to increased formation of H_2O_2, other reactive oxygen species, free radicals and GSSG. Rats fed on a low protein diet show increased susceptibility to 98% oxygen; this effect was associated with deficiency of dietary sulfur amino acids (Meister and Anderson 1983). Potentially significant connections between GSH and carcinogenesis have attracted attention. GSH protects against toxic damage in several ways; by binding directly to the toxicant, by maintaining protein thiols in a reduced state, and by reducing active oxygen species or free radicals generated by chemical or physical agents. Direct conjugation with electrophilic organic compounds is catalyzed by glutathione-s-transferases.

GSH has a key function in the hepatic detoxification of various xenobiotics, and appears to be involved in the mechanism behind acute liver effects seen shortly after injections of Cd (Dudley and Klaassen 1984). Rats that were pretreated with agents, which depleted the GSH concentration in liver cells, were much more susceptible to Cd administered intravenously at 1.3 or 2.0 mg/kg (Dudley and Klaassen 1984). GSH-depleted animals had high mortality and revealed profound morphological and biochemical signs of liver toxicity. In control rats, given Cd alone, there was no mortality and only minor morphological and biochemical changes were seen. Experimental modulation of cellular GSH levels in mice has been used to explore the role of GSH in Cd induced toxicity and indicated that the intracellular GSH function is to offer

protection against Cd toxicity (Singhal et al. 1987). GSH forms complexes with heavy metals, and protects cells against metal toxicity. This tripeptide provides a first line of defense against Cd before induction of MT synthesis. Thus, GSH has a protective function in relation to acute effects of Cd on the liver.

There are many methods for the determination of GSH (Meister and Anderson 1983). In early work, total nonprotein thiols were determined by iodometric titration or by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). Enzymatic determination of GSH has been accomplished using glyoxalase and GSSG reductase. Methods using GSH S-transferase are also available. Many column chromatographic procedures have been described; most are lengthy. HPLC is more rapid. Automated spectrophotometric method for determining GSH was used by Teare et al., recently (1993).

Metallothionein (MT)

The toxic effects of Cd are generally thought to be caused by "free" Cd ions; that is, Cd not bound to MT or other proteins (Goyer et al. 1989). However, Cd bound to MT may have the capacity to directly damage renal tubular membranes during uptake (Suzuki and Cherian 1987; Dorian and Klaasen 1995). Free Cd ions may produce a number of adverse effects, including inactivation of metal-dependent enzymes, activation of calmodulin, and initiation of the production of active oxygen species (Palmer et al. 1989; Waalker and Goering 1990).

MT has the capacity to bind Cd and exchange Cd for other metals *in vitro*. MT is a unique, low molecular weight, intracellular metalloprotein. Its synthesis is induced by a number of metals as well as various other environmental factors. Parenteral administration of Cd or zinc(Zn) is the most effective means of inducing MT synthesis. Mammalian MT contains 61 amino acids, of which 20 are cysteine, with no aromatic amino acids or histidine. The low

molecular weight protein contains no free SH-groups and disulfide bridges *in vivo* and always binds metals (Elinder and Nordberg 1985). MT binds five to seven heavy metal atoms through two clusters of thiolate bonds. The strength of binding can vary by as much as six orders of magnitude depending on the ion involved. MT-1 and MT-2 are the major forms of MT.

Parenteral administration of Cd or Zn is the most effective means of inducing MT synthesis. The most marked response is seen in the liver, but also in kidneys, pancreas, and to a lesser extent in other tissues. Shortly (2 to 3 hr) after parenteral administration of Cd to rodents and mice, most of the Cd in the liver is bound to high molecular weight proteins in the cytosol (Goering and Klaassen 1983), but after 8 hr, more than 80% of the Cd present in liver cell cytoplasm is already bound to MT (Nordberg et al. 1971). Although MT is mainly present in the cytoplasm of renal and hepatic cells, induction of MT results in its presence in cell nucleus (Danielson et al. 1982). It has been suggested that there always is a small amount of thionein (MT without metal ions) presented in the cytoplasm of the cells (Cherian and Nordberg 1983).

The biological function of MT under physiological conditions is still not completely understood. A high content of Zn- and Cu-containing MT in the newborns makes it possible to assume that MT serves as a storage protein for these metals in early life (Bremner et al. 1977). Experiments with adult animals also indicated that MT may serve as a reservoir for Zn, releasing the metal when the animal is in need of Zn for the various cellular processes in the body (Whanger and Ridlington 1982). It has been shown *in vitro* that Zn-MT can serve as a Zn donator to Zn-requiring apoenzymes, thereby reestablishing enzyme activity (Udom and Brady 1980).

MT plays a major role in the metabolism and toxicity of Cd. It sequesters Cd in the cells and thereby acts as a detoxifying agent. This toxic trace element was found to accumulate *in*

vivo as a Cd-MT complex which is nontoxic within the cell. *In vivo* protection against toxic doses of Cd was found to be enhanced by prior exposure to lower dose of Cd or by other treatments, such as with glucocorticoids, which induce MT synthesis (Enger 1988). In addition to acting as a detoxifying agent, MT also serves as a carrier of Cd between tissues, mainly from the liver to the kidney where Cd, which has been released from degraded MT, exerts toxic effects.

The most common techniques used in the preparation and isolation of MT are gel filtration, chromatography, and isoelectric focusing. Salt and/or organic solvent fractionation and heat precipitation procedures have also been used. Separation techniques based on the charge properties of MT, such as ion-exchange chromatography and isoelectric focusing, have shown that different forms of MT often exist in the human and horse kidney, rabbit, human and rat liver (Buhler and Kagi 1974; Chen and Ganther 1975; Nordberg et al. 1972; Pulido et al. 1966). Indirect methods, using *in vitro* binding of radioactive mercury or cadmium to MT, have been used to estimate MT concentration in various tissues. Other methods which have been used for determination of MT are an electrochemical method and high-performance liquid chromatography coupled with atomic absorption spectrophotometry. Radioimmunoassay for determination of MT is also used. This has increased sensitivity in the determination of small amounts of MT so that it is possible to measure very low concentrations of MT in plasma and urine (Tohyama and Shaikh 1981).

Sulfur Containing Amino Acids

Sulfur containing amino acids such as cysteine, cystine and methionine also play an important role in Cd toxicity. Cysteine can reverse the effects of Cd. Cd, administered subcutaneously, causes complete necrosis of the testis in mice. This effect can be prevented

by the administration of cysteine (Gunn et al. 1966). Pretreatment with cysteine, which increased the liver GSH levels, markedly decreased the liver toxicity of 3.9 mg/kg Cd given intravenously (Elinder and Kjellstrom 1986). Other sulfur containing amino acids also are related to cysteine metabolism. Cystine occurs normally in our food and it is easily converted to cysteine. A disulfide bridge (-s-s-) can be formed from the sulfhydryl groups (-SH) of two cysteine residues. The resulting disulfide is called cystine. Cystine can be reduced to cysteine. Cysteine can be synthesized from methionine and serine. Methionine is a precursor for cysteine synthesis in the activated methyl cycle. S-Adenosylhomocysteine is formed when the methyl group of S-adenosylmethionine is transferred to an acceptor such as phosphatidyl ethanolamine. S-adenosylhomocysteine is then hydrolyzed to homocysteine and adenosine. Homocysteine is an intermediate in the synthesis of cysteine. The sulfur atom of cysteine is derived from homocysteine, whereas the carbon skeleton comes from serine.

MT and GSH constitute two major intracellular cysteine pools in rat liver. When rats were fed on a soya-bean-protein diet, low in sulfur amino acids, the hepatic GSH levels of rats were decreased (Taniguchi and Cherian 1990). When rats were fed on diets lacking cysteine for 8 days, the hepatic GSH levels were decreased approximately 75% (Sendelbach et al. 1990). Pretreatment with cysteine, which increased the liver GSH levels, markedly decreased the liver toxicity of 3.9 mg/kg Cd given intravenously (Dudley and Klaassen 1984). Dietary sulfur containing amino acids are released into the blood by the splanchnic organs and removed by the liver. More than half of the sulfur amino acids taken up by the liver were used for synthesis of GSH (Garcia and Stipanuk 1992).

Lipid peroxidation and Free Radicals

Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids, and the eventual destruction of membrane lipid , producing a variety of breakdown products, including alcohols, ketones, aldehydes, and ethers (Buege and Aust 1990). Biological membranes are rich in unsaturated fatty acids and bathed in an oxygen-rich, metal-containing fluid. Therefore, it is not suprising that membrane lipids are susceptible to peroxidative attack.

Among the various effects induced by Cd in biological systems, lipid peroxidation has been observed in numerous tissues *in vitro* and *in vivo* (Manca et al. 1991). Despite the apparent incapacity of Cd to directly generate free radicals under physiological conditions, it has been demonstrated in *in vitro* that lipid peroxidation is an early intracellular event after Cd exposure.

The thiobarbituric acid (TBA) assay is the most common method to determine lipid peroxidation. The reaction product, malondialdehyde (MDA), can be extracted and its absorbance at 532 nm can be measured with a spectrophotometer (Ohkawa et al. 1979).

Free radicals are chemical species which have a single unpaired electron in an outer orbital. In such a state, the radical is extremely reactive and unstable and enters into reactions with inorganic or organic chemicals--proteins, lipids, carbohydrates--particularly with key molecules in membranes and nucleic acids. Moreover, free radicals initiate autocatalytic reactions whereby the molecules with which they react are themselves converted into free radicals to propagate the chain of damage.

Highly reactive and cytotoxic hydroxyl free radicals can cause tissue injury through a

variety of pathways including damaging DNA (reaction with thymine in DNA to produce single-strand breaks in DNA), inactivation of specific proteins (they promote sulfhydryl mediated cross-linking of labile amino acids and cause fragmentation of polypeptide chains) or via lipid peroxidation of cell membrane components, and disrupting the interstitial matrix by degradation of hyaluronic acid and collagen (Bus et al. 1976). Free radicals are present in minute amounts in various bodily tissues and they are highly reactive, thus, they are short lived.

Oxygen-free radicals have been implicated as possible etiological agents in the development of several disease or pathological conditions such as in aging (Harman 1981), arthritis (Borel 1983), carcinogenesis (Ames 1983), tumor promotion (Slaga 1983), hyperbaric oxygen toxicity (Yusa et al. 1984), radiation injury (Ewing 1983), ischemic injury to heart, brain and other tissues and the toxic action of certain chemicals (Nohl and Jordan 1983).

The relationship between cadmium toxicity and oxygen free radicals is not clear because oxygen free radicals are difficult to detect and quantitate *in vivo*. Studies have been reported that Cd exerted its direct toxic effects on testicular vascular endothelium which could lead to ischemia, hypoxia and lipid peroxidation followed by generation of highly reactive hydroxyl free radicals in the testicular tissues (Gunn and Gould 1975; Koizimi et al. 1992). No direct evidence for the involvement of these free radicals in Cd induced testicular toxicity has been established. It has been demonstrated that salicylate is non-toxic at low levels and, in fact, is a basic component of several commonly used drugs. Newly developed methodology is now available, in which, salicylate, a stable chemical trap is used which reacts with OH to form hydroxylated benzoic acids(DHBAs). These stable products can be specifically identified and quantified with HPLC-EC(Floyd et al. 1986).

Vitamin E

Vitamin E (tocopherols) functions as a potent chain-breaking antioxidant and plays a vital role against oxygen toxicity. The molecule is anchored in a highly hydrophobic hydrocarbon part of the membrane bilayer by the phytyl chain. This is just the right length to position the chromatin nucleus at the membrane interface (Kagan and Quinn 1988). In this position the chromanol ring has considerable mobility; it is able to quench free radicals and can harvest or collect the antioxidant capacity of other lipid-soluble antioxidants and water soluble reductants such as ascorbate and GSH (Packer and Kagan 1993). Because of its location and structure, it plays a pivotal role in antioxidant defense system and inhibits the spread of oxidative damage in membrane lipids and lipoproteins.

Vitamin E is a versatile scavenger of highly reactive oxygen derived metabolites (Morrissey et al. 1994). Vitamin E alone or in combination with vitamin C, A, carotenoids and various phenolic compounds is believed to play a vital role in defending against tissue oxidative injury caused by various toxicants (Liebler 1993) including Cd. Packer and Kagan (1993) concluded that the unique ability of low concentration of vitamin E acted as a membrane free radical harvesting center protected against oxygen toxicity. Thus, its antioxidant power is derived from other intracellular reductants.

Evidence is now accumulating that high vitamin E status decreased the incidence of certain forms of cancer (Block 1992; Knekt 1993), reduced mortality due to ischemic heart disease (Gey et al. 1991) and reduced the risk of cataract (Robertson et al. 1991). In addition, vitamin E protects low-density lipoprotein(LDL) from oxidation and reduces the damage caused by ischemia-reperfusion injury (Princen et al. 1992; Janero 1991).

Interactions of Cd with Copper(Cu), Iron(Fe) and Zinc(Zn)

Among the essential nutrients which have been studied for possible interactions, there is convincing evidence that this occurs between Cd and dietary intake or metabolism of Zn, Cu and Fe (Petering et al. 1971). High levels of dietary Cd have led to decreased tissue levels of Zn, Fe and Cu. There is considerable evidence that Cd interferes with absorption of Zn, Fe and Cu. Deficiencies of these elements could lower the threshold for absorption and foster the long-term toxic effects of Cd (Petering et al. 1984). It is also possible that at low levels of Cd intake, interactions in processes other than absorption become principally important because of differences in metalloprotein formation and biological turnover (Fox 1979). There are complex antagonistic interactions of Cd, Cu and Zn in animals. Both the concentration and distribution of Cd in tissues can be altered by Cu and Zn. Marginal Zn deficiency increased the incidence of Cd-induced injection site sarcomas and enhanced the progression of testicular tumors after a single high s.c. dose of Cd (Waalkes et al. 1991). Exposure of rats to Cd depresses serum Zn at all levels of Zn nutrition (Samarawickrama 1979). Se and Zn can protect against certain hepatotoxic effects of Cd. Zn deficiency in animals affects the gonad, and it has been found that Cd administration can displace a significant amount of testicular Zn. Low oral doses of Cd had adverse biological effects in rats when the dietary intake of Zn was low but had no such adverse effects when the dietary level of Zn was raised (Petering et al. 1979). Cd inhibits the absorption of Cu and Fe and affects Cu metabolism. Fe also inhibits Cd absorption (Petering et al. 1984).

Mills and Dalgarno (1972) showed that ewes fed on low levels of dietary Cd significantly increased liver Cd and decreased liver Cu and Zn. Whole blood Cu and plasma Zn levels were also depressed. In addition, $5 \mu g$ Cd/g in the diet fed to lambs depressed the Cu concentration

in liver, spleen and testes of the lambs after 191 days of treatment (Doyle et al. 1975). Cu and Fe concentration in the liver were also depressed. The decreased Cu, Zn and Fe concentrations in the tissues of many species are probably the result of decreased absorption and a depletion of the metals from tissues by Cd (Fox 1974).

Interference with Cu and Fe metabolism was shown to occur in rats given low levels of Cd orally. Cu and Fe metabolism are disturbed in the fetus and neonate by administering oral Cd at low doses to the dams during pregnancy, and reductions in Cu and Fe metabolism are associated with behavioral changes in these offsprings (Pond and Walker 1972).

Table 1 presents a simplified scheme for the relationships that have been observed between Cd and Cu, Fe, Zn. There are several general explanations of the adverse effects of dietary Cd

Tab. 1. Effects of Cu, Fe, Zn on toxicity of Cd

Minerals	Dietary intake of individual minerals		
	normal[a]	deficiency[b]	excess[c]
Cu	+	∧	-
Fe	+	∧	-
Zn	+	∧	-

[a]+ Cd affects metabolism and/or function of the mineral.
[b]∧ A deficiency of the nutrient increases the severity of Cd toxicity.
[c]- An excess of the mineral decreases the toxicity of Cd.

to antagonism of essential minerals (Fox 1974). Cd may replace Zn or some metals in an enzyme or at some other site and interfere with a specific metabolic reaction. Cd may displace an essential element or the carrier of an essential element in a transport system, thus disrupting such processes as intestinal absorption, transport and storage within the body and excretion. Data from numerous studies suggest that absorption is a critical point in many of the Cd-

mineral antagonisms.

A number of methods have been developed for trace element analysis. The most commonly used methods, at present, are atomic absorption spectrometry (AAS), electrochemical methods, neutron activation analysis, atomic emission spectrometry, atomic fluorescence spectrometry and proton-induced X-ray emissions analysis. AAS is now probably the most widely used technique for determination of metals including Cd, Zn, Cu and Fe in biological materials. The basic principle is to pass the sample into a high-temperature flame or furnace and measure the absorption from the atoms in the ground state. In practice, flame AAS should be selected instead of graphite furnace whenever possible because it is less time-consuming and also less sensitive to interference (Jorhem 1993).

Most of the different types of biological samples require a procedure by which the sample could be made into a solution before analysis by AAS. The two most commonly used techniques to accomplish this are dry ashing at a defined temperature, and wet digestion with mineral acid.

Conclusion and Statement of Thesis Problem

Cd is a well studied environmental pullutant. It is toxic to virtually every system in the animal body whether it is ingested, injected or inhaled. The testes are extremely sensitive to the toxic effects of Cd. The causal relationship between Cd-induced testicular toxicity and generation of hydroxyl free radical is not clear at this time due to the fact that it is extremely unstable, difficult to detect and quantitate *in vivo*. Na^+, K^+-ATPase, an integral part of the cell membrane plays an integral role in the active transport of Na^+ and K^+ across cell membrane. Thus, Na^+, K^+-ATPase plays a vital role in the major communication system linking the

extracellular signals to the intracellular medium, not only of neural tissues but also of non-neural tissues. Studies have demonstrated that Cd inhibited ATPase enzymes system in animal brain, liver and kidneys. There are, at present, no reports of studies which examine the relationship between lipid peroxidation, hydroxyl free radical generation and Na^+, K^+-ATPase activity in Cd-induced testicular toxicity. Vitamin E is known to be involved in the overall cellular anti-oxidant defense system and it is possible that vitamin E protects against Cd-induced testicular toxicity. The first objective of the proposed study was to characterize Cd-induced changes in 1) ATPase enzyme activies; 2) GSH content and the ratio of GSH/GSSG; 3) lipid peroxidation; 4) hydroxyl free radical generation and 5) to determine whether or not pretreatment with vitamin E will offer protection against Cd-induced hydroxyl free radicals generation in mouse testis.

MT is an unique low molecular weight intracellular protein. Its synthesis is induced by a number of metals including Cd as well as various other environmental factors. Mammalian MT contains 61 amino acids, of which 20 are cysteine. MT plays a major role in the metabolism and toxicity of Cd. It sequesters Cd in the cells and thereby acts as a detoxifying agent. GSH is a nonprotein thiol which is involved in cellular protective mechanisms which modulate responses to various toxic agents including Cd. The structure of GSH was established as γ-glutamylcysteinylglycine. The most important source of cysteine is dietary intake, either directly as a constituent of dietary protein or indirectly as methionine which is capable of providing cysteine equivalents for incorporation into GSH.

Among the essential nutrients which have been studied for possible interactions, there is convincing evidence that this occurs between Cd and dietary intake or metabolism of Zn, Cu and Fe. The nutritional status greatly influences the metabolic fate and toxicity of Cd. However, the influence of dietary sulfur containing amino acids such as methionine and cystine

on Cd-induced toxicity has not yet been characterized. Therefore, the second objective of the proposed study was to determine whether or not sulfur containing amino acids such as methionine and cystine in the diet will have an influence on Cd-induced biochemical changes in major target organs and to study their influence on interactions between Cd and other metals such as Cu, Fe and Zn.

REFERENCES

Ames B.N. (1983) Dietary carcinogens and anticarcinogens, oxygen radicals and degenerative diseases. Science 221:1256-1264

Andreuzzi P. and Odescalchi C.P. (1958) Experimental acute intoxication from cadmium chloride in the rabbit. Boll Soc Ital Biol Sper 34:1376-1379

Axelsson B. and Piscator M. (1966) Renal damage after prolonged exposure to cadmium. An experimental study. Arch Environ Health 12:360-373

Aylett B.J. (1979) The chemistry and bioinorganic chemistry of cadmium. Webb M.(ed). The chemistry, biochemistry and biology of cadmium. Elsevier/ North-Holland Biomedical Press, Amsterdam, Netherlands pp. 1-44

Bernard A., Roels H., Hubermont G., Buchet J.P., Masson P.L. and Lauwerys R.R. (1976) Characterization of the proteinuria in cadmium-exposed workers. Int Arch Occup Environ Health 38:19-30

Block G. (1992) The data support a role for antioxidants in reducing cancer risks. Nutri Rev 50:207-213

Borel J. P. (1983) Radicals libries oxygenes, tissue conjontifet inflammation (1983) Pathol Biol 31:8-10

Bremner I., Williams R.B. and Young B.W. (1977) Distribution of copper and zinc in the liver of the developing sheep foetus. Br J Nutr 38:87-92

Buege J. and Aust S.D. (1990) Microsomal lipid peroxidation. Meth Enzyme 186:302-311

Buhler R.H.O. and Kagi J.H.R. (1974) Human hepatic metallothioneins. FEBS Lett 39:229-234

Bus J.S., Cagon S.Z., Olgaard M, Gibson JE (1976) A mechanism of paraquat toxicity in mice and rats. Toxicol Appl Pharmacol 35:501-513

Chen R.W. and Ganther H.E. (1975) Relative cadmium binding capicity of metallothionein and other cytosolic fractions in various tissues of the rat. Environ Physiol Biochem 5:378-388

Cherian M.G. and Nordberg M. (1983) Cellular adaptation in metal toxicology and metallothionein. Toxicology 28:1-15

Copius Peereboom-Stegeman J.H.J. and Jongstra-Spaapen E.J. (1979) The effect of a simgle sublethal adminstration of cadmium chloride on the microcirculation in the uterus of the rats. Toxicology 13:199-213

Danielson K.G., Ohi S. and Huand P.C. (1982) Immunochemical detection of metallothionein in specific epithelial cells of rats organs. Proc Natl Acad Sci 79:2301-2304

Dorian C. and Klaasen (1995) Disrepancy between the nephrotoxic potencies of cadmium-metallothionein and cadmium chloride and the renal concentration of cadmium in the proximal convoluted tubules. Toxicol Appl Pharmacol 130:161-168

Doyle J.J., Bernhoft R.A. and Sandstead H.H. (1975) The effects of a low level of dietary cadmium on blood pressure, ^{24}Na, ^{42}K and water retention in growing rats. J Lab Clin Med 86:57-64

Dudley R.E., Svoboda D.J. and Klaassen C.D. (1982) Acute exposure to cadmium causes severe liver injury in rats. Toxicol Appl Pharmacol 65:302-313

Dudley R.E. and Klaassen C.D. (1984) Changes in hepatic glutathione concentration modify cadmium-induced hepatotoxicity. Toxicol Appl Pharmacol 72:530-538

Elinder C.G. and Kjellstrom T. (1986) Cadmium and health: a toxicological and epidemiological appraisal: carcinogenic and genetic effects. (Friberg L., Elinder C.G., Kjellstrom T. and Nordberg F. Eds.). C.R.C. Press, Florida. Vol. II, pp. 206-228

Elinder C.G. and Nordberg M. (1985) Cadmium and health: a tocicological and epidemiological appraisal: Metallothionein. (Friberg L., Elinder C.G., Kjellstrom T. and Nordberg F. Eds). C.R.C. Press, Florida. Vol. I, pp.65-75

Enger M.D. (1988) Reviews of environmental contamination and toxicology: cellular toxicology. (Ware G.W. Ed.). Springer-Verlag, New York. Vol. 102, pp. 79-106

Ewing D. (1983) Synergistic damage from H_2O_2 and OH⁻ radicals in irradiated cells. Radiation Res 94:171-189

Faeder E.J., Chaney S.Q. and King L.C. (1977) Biochemical and ultrastructural changes. Toxicol Appl Pharmacol 39:473-487

Favino A. and Nazari G. (1967) Renal lesions induced by a single subcutaneous injection of cadmium chloride in rat. Lav Um 19:367-372

Floyd R.A., Henderson R., Watson J.J. and Wong P.K. (1986) Use of salicylate with high pressure liquid chromatography and electrochemical detection (LCEC) as a sensitive measure of hydroxyl free radicals. J Free Radic biol Med 2:13-18

Foster C.L. and Cameron E. (1963) Observation on the histological effects of sub-lethal doses of cadmium chloride in the rabbit. J Anat 97:281-288

Fox M.R.S. (1974) Effect of essential minerals on cadmium toxicity. J food Sci 39:321-324

Fox M.R.S. (1979) Nutritional influences on metal toxicity: cadmium as a model toxic element. Environ Health Perspec 29:95-104

Friberg L. (1950) Health hazards in the manufacture of alkaline accumulatiors with special

reference to chronic cadmium poisoning. Acta Med Scand 138:1-124

Friberg L., Elinder C.G. and Kjellstrom T. (1992) Environmental health criteria 134-cadmium. World Health Organization, Geneva. pp.24-35

Garcia R.A.G. and Stipanuk M.H. (1992) The splanchnic organs, liver and kidney have unique roles in the metabolism of sulfur amino acids and their metabolites in rats. J Nutr 122:1693-1701

Gey K.F., Puska P. Jordan P. and Moser U.K. (1991) Inverse correlation between plasma vitamin E and morlality from ischemic heart disease in cross-cultural epidemiology. Amer J Clin Nutri 53:326s-334s

Goering P.L. and Klaassen C.D. (1983) Altered subcellular distribution of cadmium following cadmium pretreatment: possible mechanism of tolerance to cadmium-induced lethality. Toxicol Appl Pharmacol 70:195-203

Goyer R.A., Miller C.R. and Zhu S.Y. (1989) Non-metallothionein-bound cadmium inthe athogenesis of cadmium nephrotoxicity in the rat. Toxicol Appl Pharmacol 101:232-244

Gross S.B., Yeager D.W., Middendrof M.S. (1976) Cadmum in liver, kidney and hair of humans, fetal through old age. J Toxicol Environ Health 2:153-167

Gunn S.A., Gould T.C. and Anderson W.A.D. (1966) Protective effect of thiol compounds against cadmium-induced vascular damage to testis. Proc Soc Exp Biol Med 122:1036-1039

Gunn S.A, Gould T.C, Anderson W.A.D. (1968) Mechanism of zinc, cysteine and selenium protection against cadmium-induced vascular injury to mouse testis. J Reprod Fert 15:65-70

Gunn S.A. and Could T.C. (1975) Vasculature of the testis and adnexa. In: Hamilton DW, Greep RO, Williams and Wilkins, Baltimore. pp. 117-142

Harman D. (1981) The aging process. Proc Natl Acad Sci 78:7124-7128

Heinrich U. (1988) Environmental toxin series 2: Cadmium. (Stoeppler M. and Piscator M. Eds.). Springer-Verlag, FRG. pp. 12-24

Itokawa Y., Nisevier K., Daijo K. and Kawamura J. (1978) Renal and skeletal lesions in experimental cadmium poisoning of rats. Environ Res 15:206-217

Janero D.R. (1991) Therapeutic potential of vitamin E against myocardial ischemic-reperfusion injury. Free Radi Bio Med 10:315-324

Jorhem L. (1993) Determination of metals in foodstuffs by atomic absorption spectrophotometry after dry ashing: NMKL interlaboratory study of lead, cadmium, zinc, copper, iron, chromium, and nickel. J AOAC Intern 76:798-813

Kagan V.E. and Quinn P.J. (1988) The interaction of a-tocopherol and homologues with

other hydrocarbon chains in phospholipid bilayer dispersions. Eur J Biochem 171:661-667

Kawai M., Fukuda K. and Kimura M. (1976) Morphological alterations in experimental cadmium exposure with special reference to the onset of renal lesion. In: Nordberg GF ed. Effects and dose-response relationships of toxic metals, Amsterdam, Oxford, New York, Elsevier Science Publishers. pp. 343-370

Kinne-Saffran E., Hulseweh M., Pfaff C.H. and Kinne R.K.H. (1993) Inhibition of Na, K-ATPase by cadmium: Different mechanisms in different species. Toxicol Appl Pharmcol 121:22-29

Knekt P. (1993) Epidemiology of vitamin E: Evidence for anticancer effects in humans. (Packer L. and Fuchs J Eds.) In: vitamin E in health and disease. London: Taylor and Francis. pp. 513-527

Koizumi T., Li Z.G. and Tatsumoto H. (1992) DNA damaging acting of cadmium in leydig cells; a target cell population for cadmium carcinogenesis in rat testis. Toxicol Lett 63:211-220

Kosower N.S. and Kosower E.M. (1978) The glutathione status of cells. Int Rev Cytol 54:109-155

Lauwerys R.R., Buchet J.P., Roels H.A., Brauwers J. and Stanescu D. (1974) Epidemiological survery of workers exposed to cadmium. Arch Environ Health 28:145-148

Liebler D.C. (1993) The role of metabolism in the antioxidant function of vitamin E. Criti Rev Toxic 23:147-169

Manca D., Ricard A.C., Trottier B. and Chevalier G. (1991) Studies on lipid peroxidation in rat tissue following administration of low and moderate doses of cadmium chloride. Toxicology 67:303-323

Meister A. and Anderson M.E. (1983) Glutathione. Annu Rev Biochem 52:711-760

Merali Z., Kacew S. and Singhal R.L. (1974) Response of hepatic carbohydrate and cyclic AMP metabolism to cadmium treatment in rats. Can J Physiol Pharmacol 53:174-184

Mills C. and Dalgarno A. (1972) Copper and zinc status of ewes and lambs receiving increased dietary concentrations of cadmium. Nature 239:171-175

Moore W., Stara J.F., Crocker W.C., Malanchuk M. and Iltis R. (1973) Comparison of 115m cadmium reteintion in rats following different routes of administration. Environ Res 6:473-478

Morrissey P.A., Quinn P.B. and Sheehy P.J.A. (1994) Newer aspects of micronutrients in chronic disease: Vitamin E. Proc Nutri Soci 53:571-582

Nohl H. and Jordan W. (1983) OH-generation by adriamycin semiquinone and H_2O_2; an explanation of the cardiotoxicity of anthracycline antibiotics. Biochem Biophys Res Commun 114:197-205

Nomiyama K. and Nomiyama H. (1982) Tissue metallothioneins in rabbits chronically exposed to cadmium, with special reference to the critical concentration of cadmium in the renal cortex, in biological roles of metallothionein. (Foulkes, E.C. Ed.). Elsevier, New York. pp. 47-67

Nordberg G.F., Piscator M. and Lind B. (1971) Distribution of cadmium among protein fractions of mouse liver. Acta Pharmacol Toxicol 29:456-470

Nordberg G.F., Nordberg M., Piscator M. and Vesterberg O. (1972) Separation of two forms of rabbit metallothionein by isoelectric focusing. Biochem J 126:491-498

Ohkawa H., Ohishi N. and Yagi K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analyt Biochem 95:351-358

Packer L. and Kagan V.E. (1993) Vitamin E: The Antioxidant Havesting Centre of Membranes and Lipoproteins. (Packer L. and Fuchs J. Eds.). In: Vitamin E in Health and Disease. Marcel Dikker Inc., New York. pp.179-192

Palmer K.C., Mari F. and Malian M.S. (1989) Cadmium-induced Acute Lung Injury. Environ Res 41:568-584

Petering D.H., Loftsgaarden J., Schneider J. and Fowler B. (1984) Metabolism of cadmium, zinc and copper in the rat kidney: the role of metallothionein and other binding sites. Environ Health Perspect 54:73-81

Petering D.G., Johnson M.A. and Stemmer K. (1971) Studies of zinc metabolism in the rat. Arch Environ Health 23:93-96

Petering D.G., Choudhury H. and Stemmer K. (1979) Some effects of oral ingestion of cadmium on zinc, copper, and iron metabolism. Environ Health Perspect 28:97-106

Pond W.G. and Walker E.F. (1972) Cadmium induced anemia in growing rats: prevention by oral or parenteral iron. Nutr Rept Int 5:365-369

Princen H.M.G., Van Poppel G., Vogelezang C., Buytenkek R. and Kok F.J. (1992) supplementation with vitamin E but not B-carotene in vivo protects low density lipoprotein from lipid peroxidation in vitro. Atheroscle Thromb 12:554-562

Pulido P., Kagi J.H.R. and Vallee B.L. (1966) Isolation and some properties of human metallothionein. Biochem 5:1768-1777

Ragan H.A. and Mast T.J. (1990) Reviews of environmental contamination and toxicology. (Ware E.W. Ed.). Springer-Verlag, New York. pp. 1-22

Rajanwa B., Chapatwala K.D., Vaishnav D.D, Dasaiah D. (1981) Changes in ATPase activities in tissues of rats fed on cadmium. J Environ 2:1-9

Recht P. (1978) Criteria (Dose/Effect Relationships) for cadmium. Commission of European Communities, Pergamon Press, Great Britain . pp. 13-15

Reed D.J. and Beatty P.W. (1980) Biosynthesis and regulation of glutathione: toxicological implications. Rev Biochem Toxicol 2:213-238

Robertson J. McD., Donner A.P. and Trevithick J.R. (1991) A possible role for vitamins C and E in Cataract Prevention. Amer J Clin Nutri 53:3465-3515

Samarawickrama G.P. (1979) The chemistry, biochemistry and biology of cadmium: Biological effects of cadmium in mammals (Webb M. Ed.). Elsevier/North-Holland Biomedical Press, New York. pp. 341-421

Sendelbech L.E., White C.A., Howell S., Gregus Z. and Klaassen C.D. (1990) Effects of sulfhydryl-deficient diets on hepatic metallothionein, glutathione, and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) levels in rats. Toxicol Appl Pharmacol 102:259-267

Shaikh Z.A. and Lucis O.J. (1972) Biological difference in cadmium and zinc turnover. Arch Environ Health 24:410-418

Singhal R.K., Anderson M.E. and Meister A. (1987) Glutathione, a first of defense against cadmium toxicity. FASEB J 1:220-223

Slaga T.J. (1983) Overview of tumor promotion in animals. Environ Health Perspect 50:3-14

Sporn A., Dinu I. and Stoenescu L. (1970) Influence of cadmium administration on carbohydrate and cellular energetic metabolism in the rat liver. Rev Roum Biochim 7:299-305

Stowe H.D., Wilson M. and Goyer R.A. (1972) Clinical and morphological effects of oral Cd toxicity in rabbits. Arch Pathol 94:389-405

Suzuki C.A.M. and Cherian M.G. (1987) Renal toxicity of cadmium-MT and enzymuria in rats. J Pharmacol Exp Ther 240:314-319

Taniguch M. and Cherian M.G. (1990) Ontogenic changes in hepatic glutathione and metallothionein in rats and the effect of a low-surphur-containing diet. Br J Nutr 63:97-103

Teare J.P., Punchard N.A. and Poewll J.J. (1993) Automated spectrophotometric method for determining oxidized and reduced glutathione in liver. Clin Chem 39:686-689

Tohyama C. and Shaikh Z.A. (1981) Metallothionein in plasma and urine of cadmium exposed rats determined by a single-antibody radioimmunoassay. Fundam Appl Toxicol 1:1-7

Udom A.O. and Brady F.O. (1980) Reactivation in vitro of zinc requring apoenzymes by rat liver zinc thionein. Biochem J 187:329-335

Waalkes M.P. and Goering P.L. (1990) MT and other cadmium-binding proteins: Recent developments. Chem Res Toxicol 3:281-288

Waalkes M.P., Kovatch R. and Rehm S. (1991) Effect of chronic dietary zinc deficiency on cadmium toxicoty and carcinogenesis in the male Wistar (Hsd: (w1) BR) rat. Toxicol Appl Pharmacol 108:448-456

Waalkes M.P., Oberdoster G. (1990) Cadmium carcinogenesis. (Foulkes E.C. Ed.). C.R.C. Press, Boca Raton. pp. 129-158

Whanger P.D. and Ridlington J.W. (1982) Role of metallothionein in zinc metabolism, in Biological Roles of Metallothionein. (Foulkes E.C., Ed.). Elsevier, Amsterdam. pp. 263-277

Yusa T., Crapo J.D. and Freeman B.A. (1984) Hyperoxia enhances lung and liver nuclear superoxide generation. Biochim Biophys Acta 798:167-174

CHAPTER II

DETERMINATION OF HYDROXYL FREE RADICAL FORMATION IN THE TESTES OF CADMIUM-TREATED MICE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ABSTRACT

An application of high performance liquid chromatography (HPLC) with electrochemical detection (EC) to investigate the hydroxyl free radical generation in the testes of cadmium-treated mice is described. Salicylate was used to trap hydroxyl free radicals in vivo in the testes of cadmium-treated mice. Using this HPLC-EC method, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihyroxybenzoic acid (2,5-DHBA), the products formed by hydroxyl radical addition to salicylate, were separated and quantitated. It was found that the concentrations of both 2,3-DHBA and 2,5-DHBA in the testes of cadmium-treated mice were significantly higher than that without the treatment of cadmium. The results of this study suggest that the testicular tissue damage induced by cadmium could be partly attributed to an increase in the production of hydroxyl free radicals.

INTRODUCTION

Hydroxyl free radicals and superoxide are two oxygen radicals which have been implicated as possible etiological agents in the development of several disease or pathological conditions such as in aging (Harman 1981), arthritis (Borel 1983),carcinogenesis (Ames 1983), tumor

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promotion (Slaga 1983), hyperbaric oxygen toxicity(Yusa et al. 1984), radiation injury (Ewing 1983), ischemic injury to heart, brain and other tissues and the toxic action of certain chemicals (Nohi and Jordan 1983; Bus et al. 1976). Superoxide radicals are not as reactive as hydroxyl radicals in aqueous solution. In comparison, hydroxyl free radicals are highly reactive and short lived in both chemical (Ye and Schuler 1990; Ye 1992) and biological systems (Fridovich 1978), and present at very low concentrations in tissues in normal conditions. Hydroxyl free radicals can cause tissue injury through a variety of pathways including damaging DNA, inactivating specific proteins or via lipid peroxidation of cell membrane components, and disrupting the interstitial matrix by degradation of hyaluronic acid and collagen (Udassin et al. 1991).

Cadmium is a toxic trace metal. Occupational and environmental pollutants are the main sources of cadmium exposure. Cadmium is toxic to virtually every system in the animal body, whether ingested, injected, or inhaled. The toxic effects of exposure to cadmium include anemia, dermatitis, testicular degeneration or atrophy, reduced growth rate, liver and kidney damage, cardiovascular disorders, pulmonary edema and emphysema, teratogenic malformations and increased mortality (Friberg and Elinder 1992; Elinder and Kjellstrom 1986). Cadmium induced lipid peroxidation has been observed in numerous tissues either *in vivo* or *in vitro* (Gabor et al. 1978; Shukla et al. 1987). The relationship between cadmium toxicity and hydroxyl free radicals generation is not clear because hydroxyl free radicals is difficult to detect and quantitate *in vivo*. Salicylate is non-toxic at a low concentration and has been used to trap hydroxyl free radicals in animals (Udassin et al. 1991; Floyd et al. 1986). Two main products are 2,3-DHBA and 2,5-DHBA formed by hydroxyl addition to salicylate (Floyd et al. 1984).

The objective of this study was to use salicylate as a free radical trapping agent to detect

hydroxyl free radicals *in vivo* and provide a direct evidence for pathophysiological role of the hydroxyl free radicals in the testis toxicity caused by cadmium.

MATERIAL AND METHODS

Chemicals and Reagents:

All the reagents were of analytical grade. Cadmium chloride, trichloroacetic acid (TCA), sodium salicylate and its monohydroxylated products, 2,3-DHBA and 2,5-DHBA were obtained from Sigma Chemical Company (St. Louis, MO). Water filtered through a Millipore Milli-Q system (Millipore, Milford, MA, USA) was used.

Animal Preparation:

Male, CD-1 mice (Charles River Breeding Labs) with body weights of 30-35g were used in all experiments. They were housed in groups of 5 mice/cage, kept at a constant room temperature and humidity maintained on a controlled environment with a 12h light : 12h dark cycle. Food and water were provided *ad libitum*. After one week adaption, the mice were divided into two groups of 5 mice each. The individual mice within the treatment group received subcutaneous injection of cadmium chloride at 2mg/Kg a day for 5 days. The individual mice within the control group was given an equal volume of saline.

HPLC Assay:

The HPLC system (Waters, Division of Millipore, Milford, MA, USA), which included a 501 HPLC pump, an U6K injector with a 500 ul sample loop, a 460 electrochemical detector with a glassy carbon working electrode, an auxiliary electrode and a Ag/AgCl reference

electrode was used in this study. Data acquisition and processing were accomplished by a Waters Baseline 810 chromatographic work station which included a NEC PowerMate SX/16 computer and a system interface. The column used was a Hibar RT Lichrosorb-RP-18 (10um, 250x4mm)(E. Merck, Gibbstown, NJ, USA). The voltage in the electrochemical detector was set at 0.8V. The injection volume was 50µl and the flow rate of the mobile phase was 0.8ml/min. The mobile phase was 0.03M citric acid and 0.03M acetic acid, and prepared by titration with sodium hydroxide to pH 3.0 followed by titration with sodium acetate to a final pH of 3.6.

The calibration curves of the DHBAs were generated with various concentrations of 2,5-DHBA and 2,3-DHBA standards in 10% TCA solution. The concentrations of the two DHBAs in the tissue extracts were determined by comparison of the peak areas of the extracts with that of the calibration curves of 2,5-DHBA and 2,3-DHBA.

Sample Preparation:

After 5 days of cadmium or saline treatment, sodium salicylate (100mg/kg, i.p.), was administered to the individual mouse, 30 min prior to sacrifice by cervical dislocation. The testis was removed immediately, weighed and placed in ice-cooled 10% TCA solution (1.5ml/200mg tissue). The testis was homogenized in a polytron homogenizer (Polytron model #PT 10/35, Brinkman Instruments, Switzerland), and centrifuged at 2,000 RPM at room temperature for 10 min. The supernatants were filtered with a 0.22µm Millipore filter, and then 50µl of this solution was injected into the HPLC system.

Recovery Assay:

2,3-DHBA and 2,5-DHBA solutions at various concentrations were spiked into the blank

tissues and then extracted with the same procedure described above. The concentrations of the DHBAs in the tissue extracts were determined by comparison of the HPLC peak areas obtained on the extract to that of a standard curve obtained by diluting 2,3-DHBA and 2,5-DHBA in TCA.

Statistical Analysis of the Data:

The statistical analysis of the experimental data of 2,3-DHBA and 2,5-DHBA in testes were carried out using Student t test. The results were considered significant at P<0.05.

RESULTS

As shown in Figure 1a, 2,5-DHBA and 2,3-DHBA were not detected in the blank extracts of testes from mice which were not treated with either salicylate alone and/or cadmium. Figure 1b shows the chromatogram of the extracted sample from a mouse, given only salicylate (100mg/kg). Figure 1c shows the chromatogram of the extract of a sample from the cadmium-treated mouse. The chromatographic peaks of 2,5-DHBA and 2,3-DHBA in testes of the control group (Figure 1b) are much smaller than those of testes of cadmium-treated group (Figure 1c). Figure 1d shows the chromatogram of testicular tissue extracts spiked with 1 μM of 2,5-DHBA and 2,3-DHBA.

Four consecutive standard curves for 2,3- and 2,5-DHBA demonstrated a good linear relationship between concentration and peak area. The calibration curves could be described by the regression equations: $Y = 8.1242 \pm 0.000157979X$ ($r = 0.99534$) for 2,3-DHBA and $Y = 19.4533 \pm 0.000169515X$ ($r = 0.99963$) for 2,5-DHBA. The recoveries of 2,3-DHBA and 2,5-DHBA were 91.4% and 92.1%, respectively.

Figure 2a and 2b present the mean ± SE (SE, standard error) concentrations of 2,3-DHBA and 2,5-DHBA in the testes of the control (saline) and cadmium-treated mice respectively. The mean ± SE of 2,3-DHBA and 2,5-DHBA in the control group were 36.34 ± 1.11 and 161.4 ± 68.33 nM/100mg tissue, respectively. The mean ± SE of 2,3-DHBA and 2,5-DHBA in the cadmium treated group were 73.02 ± 6.05 and 671.32 ± 42.12 nM/100mg tissue, respectively. The changes in the concentrations of 2,3-DHBA and 2,5-DHBA in the cadmium treated group in comparison with that in the control were 2.01 and 4.16 times, indicating significant increases.

The experiments were carried out to determine the possibility of further metabolism of the adducts, 2,5-DHBA and 2,3-DHBA, in testes. Testicular tissues were stored at 4°C for one week. The concentrations of 2,3-DHBA and 2,5-DHBA in these samples were determined every day by HPLC. As shown in Table 1, there was no significant change in the concentrations of DHBAs in the testicular tissue homogenates stored for 24 hours.

DISCUSSION

Superoxide and hydroxyl free radicals are two oxygen derived metabolites which are present usually only at very low concentrations in biological systems. They may either directly or indirectly contribute to the development of several pathological conditions (Fridovich 1978).

Measurement of hydroxyl free radical formation in biological systems is difficult because the hydroxyl free radical is highly reactive with most organic molecules (with rate constants of 10^9 to 10^{10} $M^{-1} \cdot s^{-1}$) and its lifetime in biological matrices is extremely short (≤ 1 nanosecond) (Buxton et al. 1988; Karam et al. 1991). With the aid of electron paramagnetic

resonance(ESR) spectroscopy in combination with spin trapping, it has become possible to detect the presence of hydroxyl free radicals. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) has been used to detect hydroxyl free radicals in isolated tissues (Kramer et al. 1987). There are many advantages to this technique but its usefulness in the study of hydroxyl free radicals in biological system has been limited. For one, the use of ESR spin trapping is limited by relative low sensitivity. A major limitation is the metabolism and instability of spin adducts *in vivo* leading to reduction of the stable spin trap-radical adduct and subsequent loss of the spectra (Samumi et al. 1986). Because of these limitations, as well as the high cost of an ESR spectrpmeter, many investigators have been using an alternative technique to detect hydroxyl free radical formation in biological systems.

The use of salicylate is based on the ability of the hydroxyl free radical to attack and add to aromatic compounds. Hydroxyl radical adds to salicylate with a reported rate constant of $1.2 \times 10^{10} M^{-1} s^{-1}$ (Maskos et al. 1992). Under physiological conditions, the major products of ·OH attack on salicylate are 2,3-DHBA, 2,5-DHBA and catechol (Fig. 3).

Using salicylate with HPLC-EC as a sensitive measure of hydroxyl free radicals in adriamycin treated rats, Floyd et al. (1986) indicated that the HPLC-EC method was useful to measure hydroxyl free radical production *in vivo* and supported the theory that adriamycin induced hydroxyl free radical generation in tissues. It is quite clear that the selectivity of separation of HPLC in combination with the specificity and high sensitivity of EC offers excellent advantages as an analytical technique. The results showed that there was a significant increase of DHBAs from salicylate as a resultant of adriamycin treatment (Floyd et al. 1986).

2,5-DHBA was the only product formed in an enzymatic salicylate metabolism *in vivo* (Grootveld and Halliwell 1986). A previous study of the reaction of hydroxyl free radicals with

salicylate *in vitro* indicated that both 2,5-DHBA and 2,3-DHBA were formed in almost equal amounts (Grootveld and Halliwell 1986). Our results indicated that the ratio of 2,5-DHBA and 2,3-DHBA was 9.19 and the increases of 2,5-DHBA and 2,3-DHBA were 4 and 2 folds in the testes of cadmium-treated mice. This result demonstrates that the testis toxicity caused by cadmium is related to the formation of hydroxyl free radicals. While the detailed mechanism of cadmium-mediated hydroxyl free radicals generation remains to be investigated, one possibility may involve the interaction of cadmium with ferritin and other iron-containing proteins, resulting in the release of iron. The released iron reacts to form hydroxyl free radicals via the Fenton reaction as suggested previously (Liochev and Frederick 1994).

Cadmium exerts its toxic effects on testicular vascular endothelium which could lead to ischemia, hypoxia and lipid peroxidation followed by generation of highly reactive hydroxyl free radicals in the testicular tissues (Aoki and Hoffer 1978; Koizumi et al. 1992). The high content of polyunsaturated fatty acids in the testis rendered this organ particularly susceptible to peroxidative damage (Omaye and Tappel 1975). Since thiol agents protect against testis toxicity caused by cadmium, the testis damage is apparently oxidative in nature. It is quite possible that cadmium toxicity in testes is mediated by oxygen free radicals. Our results show that there is a 4-fold and 2-fold increase in 2,5-DHBA and 2,3-DHBA, respectively, demonstrating direct evidence for the involvement of hydroxyl free radicals in cadmium toxicity *in vivo.* Hydroxyl free radicals are extremely reactive with a number of compounds, including lipids and proteins. The presence of hydroxyl free radicals in the testes provides important information necessary for further study of the mechanism of cadmium-induced testicular toxicity.

REFERENCES

Ames B.N. (1983) Dietary carcinogens and anticarcinogens, oxygen radicals and degenerative diseases. Science 221:1256-1264

Aoki A. and Hoffer A.P. (1978) Reexamination of the lesions in rat testis caused by cadmium. Biol Reprod 18:579-591

Borel J.P. (1983) Radicals libries oxygenes, tissue conjontifet inflammation (1983) Pathol Biol 31:8-10

Bus J.S., Cagon S.Z., Olgaard M. and Gibson J.E. (1976) A mechanism of paraquat toxicity in mice and rats. Toxicol Appl Pharmacol 35:501-513

Buxton G.U., Greenstock C.L., Helman W.P. and Ross A.B. (1988) Critical review of rate constants for reactions of hydroxyl radicals. Phys Chem Ref Data 17:513-886

Elinder C.G. and Kjellstrom T. (1986) Cadmium and health: A toxicological and epidemiological appraisal. C.R.P. Press, Florida. pp. 206-228

Ewing D. (1983) Synergistic damage from H_2O_2 and OH⁻ radicals in irradiated cells. Radiation Res 94:171-189

Floyd R.A., Watson J.J. and Wong P.K. (1984) Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and salicylate hydroxylation products. J Biochem Biophys Methods 10:221-235

Floyd R.A., Henderson R., Watson J.J. and Wong P.K. (1986) Use of salicylate with high pressure liquid chromatography and electrochemical detection (LCED) as a sensitive measure of hydroxyl free radicals. J Free Radic Biol Med 2:13-18

Friberg L. and Elinder C.G. (1992) Cadmium: Environmental helth criteria, W.H.O., Geneve. pp.28-35

Fridovich I. (1978) The biology of oxygen radicals. Science 201:875-884

Gabor S., Anca Z. and Bordas E. (1978) Cadmium-induced lipid peroxidation in kidney and testes. Rev Roum Biochem 15:113-117

Grootveld M. and Halliwell B. (1986) Reaction of triethylphosphine gold complexes with heme proteins. Biochim J 237:499-504

Harman D. (1981) The aging process. Proc Natl Acad Sci 78:7124-7128

Karam L.R., Bergtold D.S. and Simic M.G. (1991) Biomarkers of OH radical damage in vivo. Free Radic Res Commun 12:11-16

Koizumi T., LI Z.G. and Tatsumoto H. (1992) DNA damaging acting of cadmium in

leydig cells. a target cell population for cadmium carcinogenesis in rat testis. Toxicol Lett 63:211-220

Kramer J.H., Arroyo C.M., Dickens B.F. and Weglicki W.B. (1987) Spin-trapping evidence that graded myocardial ischemia alters postischemic puperoxide production. Free Radic Biol Med 3:153-159

Liochev S. and Frederuck I. (1994) Paraquat diaphorases in Escherichia coli. Free Radi Biol Med 16:29-33

Maskos Z., Rush J.D. and Koppenol W.H. (1992) The hydroxylation of phenylalanine and tyrosine. Arch Biochem Biophy 296:521-529

Nohl H. and Jordan W. (1983) OH-generation by adriamycin semiquinone and H_2O_2; an explanation of the cardiotoxicity of anthracycline antibiotics. Biochem Biophys Res Commun 114:197-205

Omaye S.T. and Tappel A.L. (1975) Influence of dietary thiamine on pulmonary, renal and hepatic drug metabolism in the mouse. Res Comun Chem Path Pharmacol 12:695-705

Samuni A., Carmichael A.J., Russo A., Mitchell J.B. and Riesz P. (1986) On the spin trapping and ESR detection of oxygen-derived radicals generated inside cells. Proceed Natl Acad Sci 83:7593-7597

Shukla G.S., Hussain T. and Chandra S.V. (1987) possible role of regional superoxide dismutase activity and lipid peroxides levels in cadmium neurotoxicity: in vivo and in vitro studies in growing rats. Life Sci 41:2215-2221

Slaga T.J. (1983) Overview of tumor promotion in animals. Environ Health Perspect 50:3-14

Udassin R., Ariel Y., Haskel N.K. and Chevion M. (1991) Salicylate as an in vivo free radical trap: Studies on ischemic insult to the rat intestine. Free Rad Biol Med 10:1-5

Yusa T., Crapo J.D., Freeman B.A. (1984) Hyperoxia enhances lung and liver nuclear superoxide generation. Biochim Biophys Acta 798:167-174

Ye M.Y. and Schuler R.H. (1990) Determination of oxidative products in radiolysis of halophenols with pulse radiolysis, HPLC and ion chromatography. J Liq Chromatogr 13:3369-3387

Ye M.Y. (1992) Determination of products in hydroxyl addition of phenol using HPLC. J Liq Chromatogr 15:875-884

TABLE 1:
The levels of 2,5-DHBA and 2,3-DHBA
in mouse testes homogenates during one week at 4°C.

day	2,5-DHBA level (nM/100mg tissue)	decrease (%)	2,3-DHBA level (nM/100mg tissue)	decrease (%)
1	161.46	0.00	36.34	0.00
2	159.97	0.92	35.31	2.83
3	155.67	3.58	32.97	9.27
4	149.50	7.41	32.52	10.51
5	146.51	9.26	31.05	14.56
6	134.56	16.66	30.20	16.90
7	132.32	18.25	29.76	18.11

FIGURE LEGENDS

Figure 1. Chromatograms of the testes extracts from a control mouse.

Figure 2.   Chromatograms of the testes extracts from a mouse treated with salicylate.

Figure 3. Chromatograms of the testes extracts from a mouse treated with salicylate and cadmium.

Figure 4. Chromatograms of 100nM standard of 2,5-DHBA and 2,3-DHBA in blank mouse testicular tissues. Principal peaks, 1: 2,5-DHBA; 2: 2,3-DHBA

Figure 5. Mean($\pm$SE) levels of 2,5-DHBA and 2,3-DHBA in the testicular tissues of cadmium treated mice(n=5) and control mice(n=5).

Fig. 1

Fig. 2

Time (min)

Fig. 3

Detector Response (V)

Time (min)

Fig. 4

Detector Response (V)

Fig. 5

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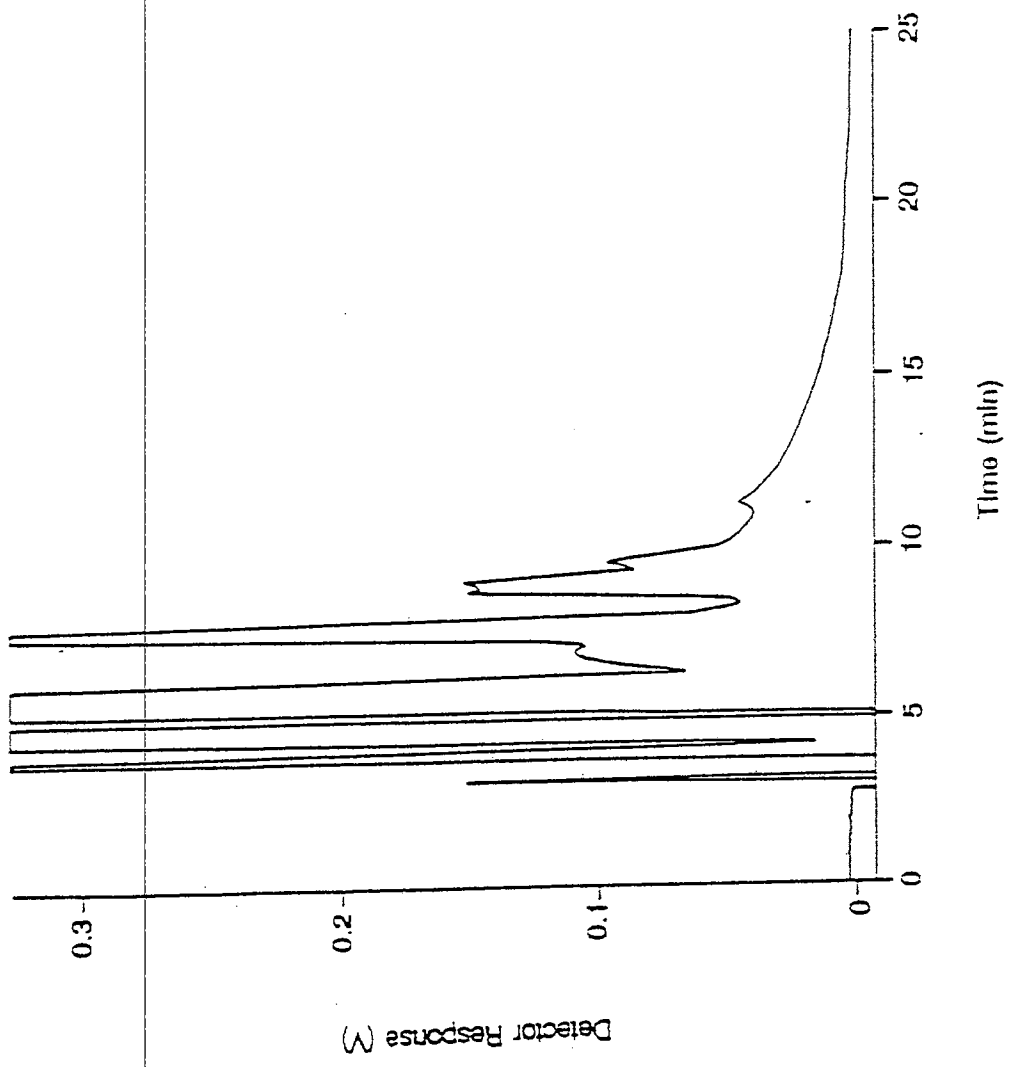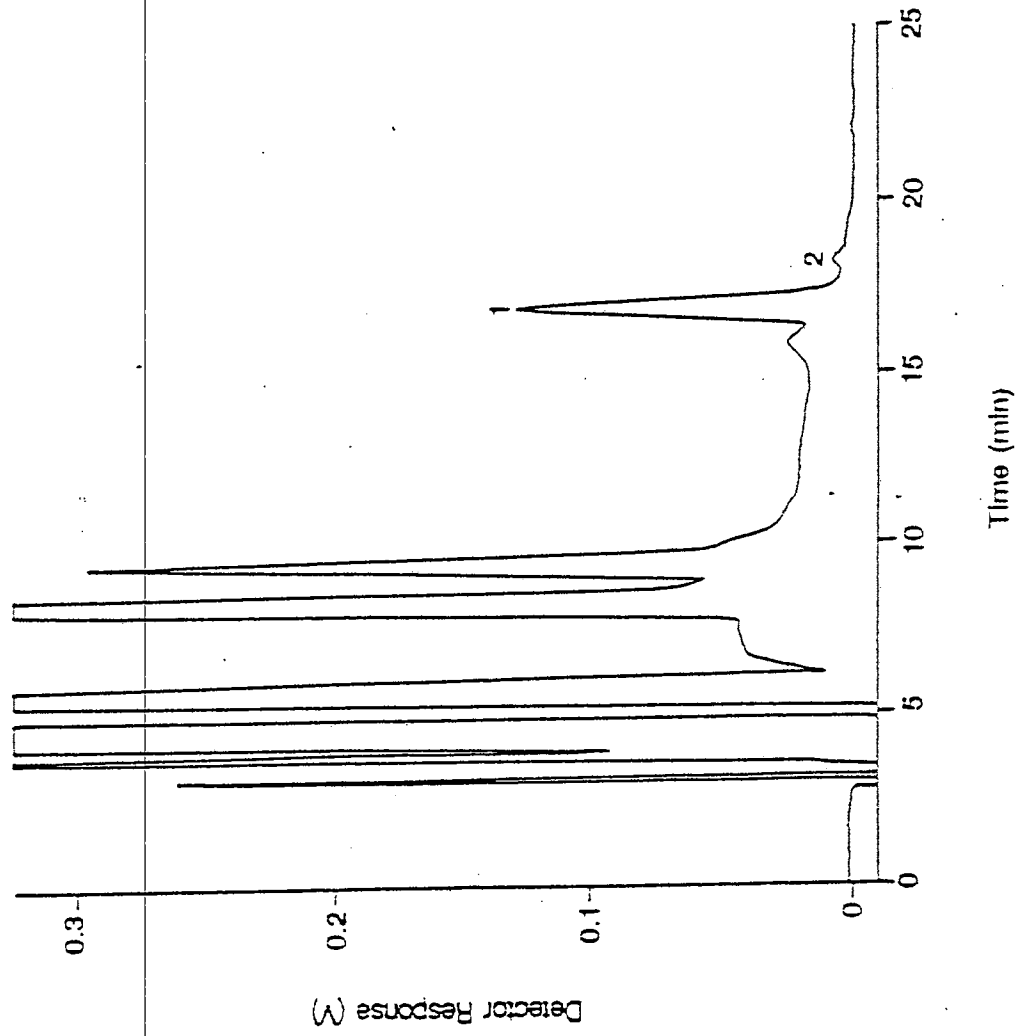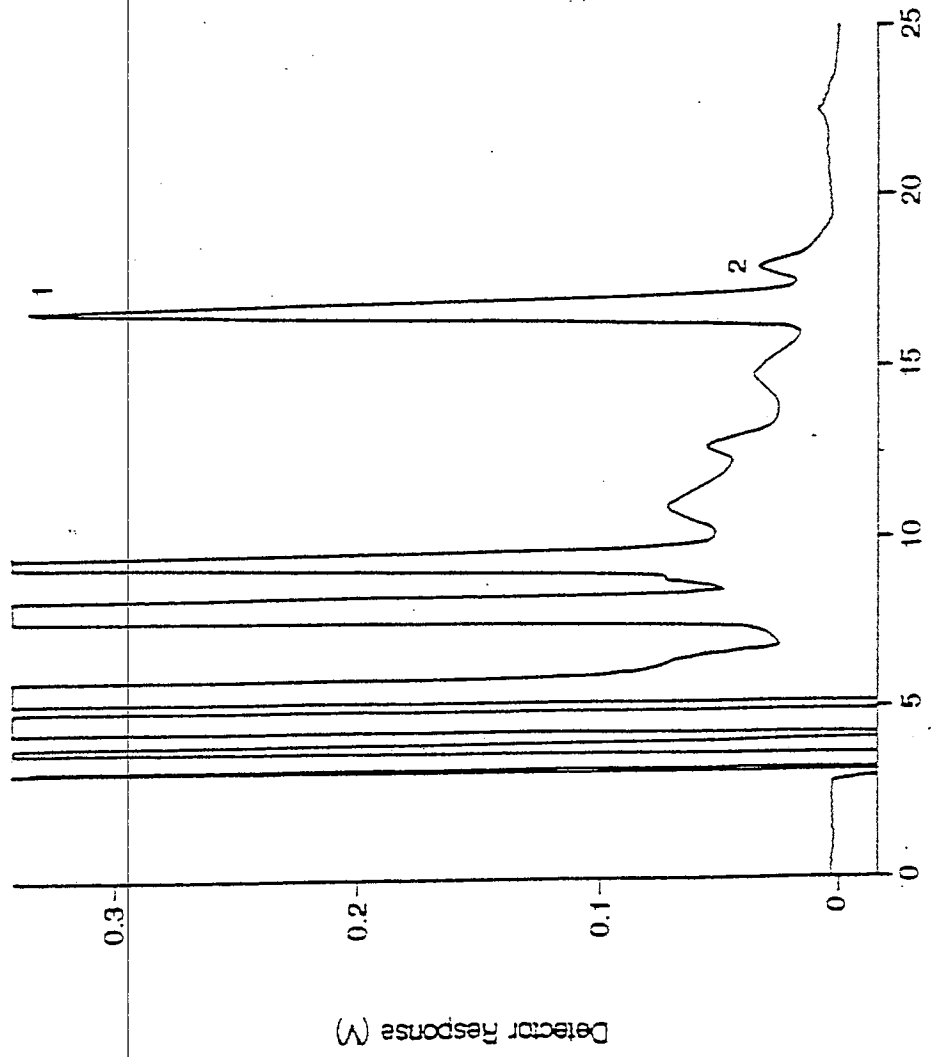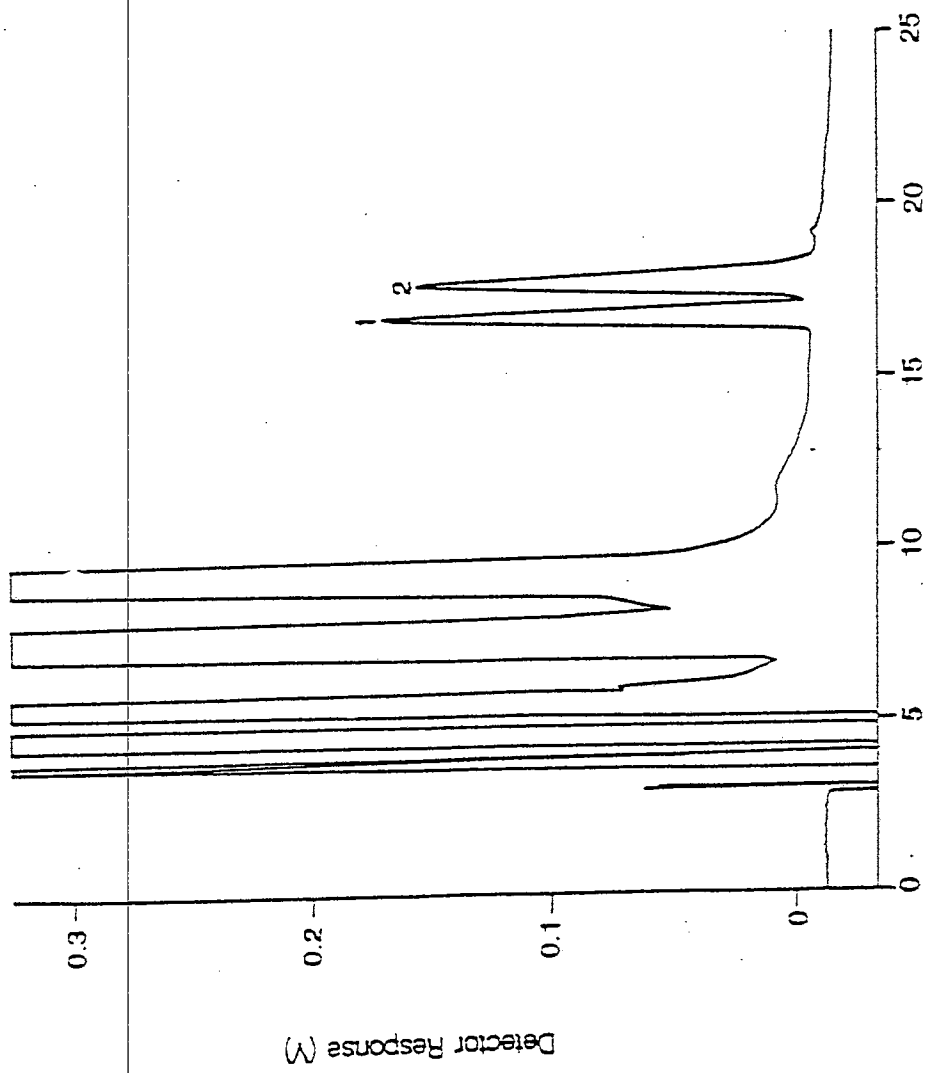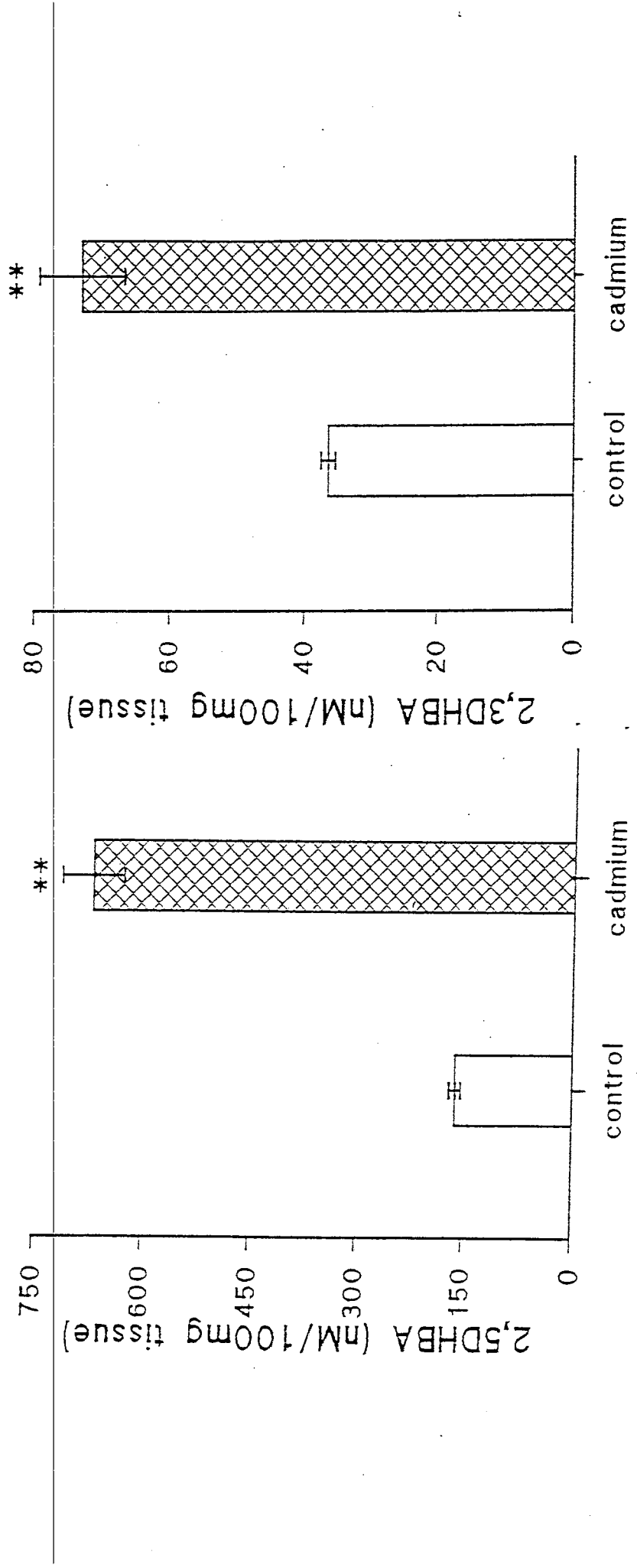
CHAPTER III

Na$^+$,K$^+$-ATPase, GLUTATHIONE, LIPID PEROXIDATION AND HYDROXYL FREE RADICALS IN CADMIUM INDUCED TESTICULAR TOXICITY IN MICE

ABSTRACT

CdCl$_2$-induced biochemical changes were characterized in male, CD-1 mouse testes. CdCl$_2$ inhibited the testes microsomal Na$^+$,K$^+$-ATPase activity *in vitro* and *in vivo*. The inhibitory concentration range was 30-150μm and the concentration for half maximal inhibition (IC50 value) was 90μm over 5 minutes preincubation. CdCl$_2$ (2mg/kg/day, s.c.) for 2 days significantly inhibited testes Na$^+$,K$^+$-ATPase (near 90% inhibition). The content of testicular GSH and the ratio of GSH/GSSG(oxidized glutathione) decreased in CdCl$_2$ treated groups. CdCl$_2$ increased the testicular lipid peroxidation both *in vivo* and *in vitro*. Using salicylate as a trapping agent and high pressure liquid chromatography with electrochemical detection (LCED), we measured the OH production *in vivo*. 2,5-dihydroxybenzoic acid (2,5-DHBA) and 2,3-dihydroxybenzoic acid (2,3-DHBA) as indices of hydroxyl free radicals formation significantly increased after 5 days CdCl$_2$ exposure. Pretreatment with vitamin E (20mg/kg, s.i.d., i.m., 7d) protected CdCl$_2$-induced increase in OH generation in testes. From this study, we were able to demonstrate that CdCl$_2$ induced testicular toxicity could possibly be mediated by a significant increase in hydroxyl free radicals formation and lipid peroxidation, and a reduction in GSH content and Na$^+$,K$^+$-ATPase activity. Vitamin E seems to prevent CdCl$_2$ induced increase in hydroxyl free radical generation.

INTRODUCTION

Cadmium is a toxicological contaminant (Ragan and Mast 1990) in the body and inhibits a number of enzymes (Samarawickrama 1979). This heavy metal plays an important role in pathogenesis of various systemic toxicoses including testicular necrosis and neoplasms (Heinrich 1988). Cadmium damages the testes of many mammals and produces a purplish discoloration of the testes (Gunn et al. 1966; Wahba et al. 1990).

A previous study has demonstrated the inhibition of ATPase enzymes system by cadmium in brain, liver, kidney of rat (Rajanwa et al. 1981). No studies have been reported on the effects of cadmium on testicular Na^+, K^+-ATPase *in vitro* and *in vivo*. Since Na^+, K^+-ATPase is the enzyme system associated with vital physiological functions such as cell volume regulation, osmotic pressure and maintenance of cellular integrity (Heinrich 1988), it is appropriate to investigate the effects of cadmium on testes Na^+, K^+-ATPase.

Oxygen-free radicals have been implicated as possible etiological agents in the development of several disease or pathological conditions such as in aging (Harman 1981), arthritis (Borel 1983), carcinogenesis (Ames 1983), tumor promotion (Slaga 1983), hyperbaric oxygen toxicity (Yusa et al. 1984), radiation injury (Ewing 1983), ischemic injury to heart, brain and other tissues and the toxic action of certain chemicals (Nohl and Jordan 1983). The highly reactive and cytotoxic hydroxyl radicals can cause tissue injury through a variety of pathways including damaging DNA, inactivating specific proteins or via lipid peroxidation of cell membrane components, and disrupting the interstitial matrix by degradation of hyaluronic acid and collagen (Bus et al. 1976).

The free radicals are present in minute amounts in various bodily tissues and they are highly reactive, thus, they are short lived. The relationship between cadmium toxicity and

oxygen free radicals is not clear because oxygen free radicals are difficult to detect and quantitate *in vivo*. Salicylate is non-toxic at low levels and, in fact, is a basic component of several commonly used drugs. A newly developed methodology is now available, employing a stable chemical trap, salicylate, which reacts with OH to form hydroxylated benzoic acids(DHBAs). These stable products can be specifically identified and quantified with LCED (Floyd et al. 1986).

Cadmium induced lipid peroxidation has been observed in numerous tissues either *in vivo* or *in vitro* (Gabor et al. 1978; Shukla et al. 1987). Vitamin E is involved in the overall cellular anti-oxidant defense against deleterious effects of reactive oxygen species (Preston 1991). This antioxidant has been shown to produce a significant reduction in lipid peroxidation induced by various chemicals and ischemic insult (Min et al. 1992). Cadmium has been shown to produce oxidative stress in the CNS and the pretreatment with vitamin E seemed to be beneficial against such damage (Ali et al. 1993). The objectives of the present study are to characterize $CdCl_2$-induced changes in 1) ATPase; 2) GSH content and the ratio of GSH/GSSG; 3) lipid peroxidation; 4)hydroxyl free radicals generation and 4) to determine whether or not pretreatment with vitamin E provides protection against $CdCl_2$-induced free radical generation in mouse testes.

MATERIALS AND METHODS

Reagents

All the reagents used in this study were of analytical grade. $CdCl_2$, trichloroacetic acid (TCA), sodium salicylate and its monohydroxylated products, 2,3-DHBA and 2,5-DHBA, GSH, GSSG, 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), NADPH, glutathione reductase

(GR), picric acid, vitamin E (α-tocopherol) and all biochemicals used for the enzyme assays were obtained from Sigma Chemical Company (St. Louis, MO). 2-vinylpyridine (2-VP) was obtained from Aldrich chemical Co. (Milwaukee, WI) and stored at -20°. Protein regent was purchased from Pierce Co.(Rockford, IN) and all solutions were made in deionized distilled water.

Animals

Adult, male, CD-1 mice(Charles River Breeding Labs), 30-35g body weight were used in all experiments. They were housed in groups of 5 mice/cage, kept at a constant room temperature and maintained on a controlled environment with a 12 hours light : 12 hours dark cycle. Food and water were provided *ad libitum*. After one week adaptation, the mice were divided into different groups.

Experimental Design

 Experiments 1 & 2: Effects of CdCl$_2$ on *in vivo* and *in vitro* Testicular ATPase Twenty mice were divided into 4 groups of 5 mice each. Group one served as a control. The other three were treatment groups. The individual mice within the treatment groups received subcutaneous injection of CdCl$_2$ at 0.5mg/kg/day, 2mg/kg/day, and 2mg/kg/day for two days respectively. The individual mice within the control group received subcutaneous injection of equal volumes of normal saline.

A group of 30 mice were used for preparation of testicular ATPase enzymes for *in vitro* studies.

Experiment 3: Effects of CdCl$_2$ on Testicular GSH and Hydroxyl Free Radical Generation

Mice were divided into control and different treatment groups with 11 or 10 mice in each group. The individual mice within the treatment groups received subcutaneous injection of CdCl$_2$ at 1mg/Kg/day over the whole 12 hours period and 1mg/Kg/day on 1st, 3rd, 5th, 7th, 9th and 12th days. The individual mice within the control group received subcutaneous injection of equal volume of normal saline at time intervals very similar to those of treatment group. Mice from both control and treatment groups were weighed and sacrificed. The testes from the individual mice were collected and weighed for determination of GSH, GSSG and hydroxyl free radicals.

Experiment 4: Effects of Vitamin E on CdCl$_2$ Induced Changes in Hydroxyl Free Radicals Generation in Testes   Mice were divided into four groups of 5 each. The individual mice within cadmium groups received 1mg/kg/day CdCl$_2$ (s.c.) over 7 days. The individual mouse within the vitamin E treatment groups received 20mg/kg/day vitamin E in corn oil (i.m.) 30 minutes prior to cadmium or saline administration over 7 days. Sodium salicylate (100mg/kg) used as a trapping agent for hydroxyl free radicals was injected intraperitoneally to the individual mice 30 minutes prior to sacrifice.

Experimental Precedures

Determination of ATPase Enzymes Activies

Enzyme Preparation: In *in vivo* experiment the individual mice was killed by cervical dislocation. Microsomal ATPase enzymes were prepared following previously established procedures (Akera and Brody 1969; Chen et al. 1992; Shen and Sangiah 1994). The testes were removed rapidly and placed in ice-cooled isotonic solution containing 0.25M sucrose,

5.0mM L-histidine, 5.0mM ethylene diamine tetraacetate (EDTA-Na$_2$) and 0.15% sodium

deoxycholate, pH 6.8 with Trizma base. Another group of thirty mice were used for *in vitro*

experiment. The mice were killed by cervical dislocation. The testes from 30 mice were minced

with scissors and homogenized in a polytron homogenizer (Polytron model # PT 10/35,

Brinkman Instruments, Switzerland) 1 minute duration with about 8g testes in 20 ml ice-

cooled isotonic sucrose solution. The homogenate was centrifuged for 30 min at 12,000xg.

Following centrifugation of the supernatant for 60 minutes at 100,000xg, the sediment was

suspended in 20ml of suspension solution (0.25M sucrose, 5.0mM L-histidine and 1mM

ethylene diamine tetraacetic acid, pH 7.0 with Trizma base). The pellet was resuspended in

10ml of the above suspension solution plus 10 ml of 2.0M LiBr and stirred gently for 1 hour.

The mixture was centrifuged for 60 minutes at 100,000xg. The sediment was resuspended in

20ml of suspension solution and recentrifuged. The microsomal pellets obtained were diluted

in suspension solution and stored at -70°C until used. All of the above procedures were carried

out at 2°C. Protein was determined by the method of Smith et al (1985). For *in vivo* Na$^+$, K$^+$-

ATPase assay, the testes from the individual mice from both control and treatment groups

were used.

  Determination of Enzyme Activity: ATPase activity in mouse testes was measured by the

enzymatic method (Broekhuysen et al. 1972) with modifications as previously reported (Chen

et al. 1992; Shen and Sangiah 1994). Enzyme assays were run at 37°C.

In *in vitro* experiments, the different concentrations of CdCl$_2$ were preincubated together

with 30-50μg protein for 5 minutes in 50mM Tris HCl buffer containing 5mM MgCl$_2$, 15mM

KCl, 100mM NaCl, pH 7.5. The reaction was initiated by adding ATP with the final

concentration being 3.0mM. The inorganic phosphate was determined spectrophotometrically

at 660nm, each assay being performed in duplicate. The Na^+, K^+-ATPase activity was calculated by subtracting the Mg^{2+}-activated ATPase activity from total ATPase activity. All results were corrected for blank values without enzyme. IC50 was calculated using computer software (Tallarida and Murray 1986).

Determination of GSH and GSSG

The contents of GSH and GSSG in mouse testes were determined by using modified procedures previously reported (Teare et al. 1993). Standards were prepared in water containing picric acid such that the concentrations of picric acid in the final reaction mixture was the same as for the diluted biological sample supernates (1.75mmol/L for GSSG and 0.35mmol/L for GSH). NADPH (0.03mmol/L) was dissolved in 0.125mol/L sodium phosphate buffer (pH 7.5) containing 6.3mmol/L sodium EDTA. DTNB, 6.0mmol/L, and GR solution (1 U/100μl) were also prepared in this buffer. Blanks containing picric acid only were prepared.

After different days of $CdCl_2$ treatment (experiment 3), the testes from the individual mice of both control and treatment groups after sacrifice, were removed rapidly, weighed and placed in ice-cooled 43 mmol/L picric acid, with 5 ml of picric acid reagent per gram of tissue. The testes were homogenized in a polytron homogenizer (Polytron model # PT 10/35, Brinkman Instruments, Switzerland) for 50 seconds. The homogenate was centrifuged (5,000 rpm at 4°C for 20 min) and supernate was divided into aliquots which were then stored at -70°C for use.

For GSH determination, 100 μl supernates were added to 9.9 ml of ice-cold water. For GSSG, 200 μl supernates were added to 3.6 ml of ice-cold water to which 40 μl of 2-VP was then added to mask the GSH. The samples were then left at room temperature for 2 hours.

The microplate was filled with 140 μl of NADPH reagent, 20 μl of DTNB reagent, and 20 μl of sample or standard and incubated at 30°C for 10 minutes. Then 20μl of GR is added, and the reaction monitored immediately with readings taken every 9 seconds for 5 minutes. For GSSG, for which the reaction time is slower, initial readings were delayed for 1 minute, with readings then taken every 15 seconds for 7 minutes so that in both assays, the reaction was monitored over the linear part of the curve.

Thiobarbituric Acid (TBA) Assay

TBA assay was used to determine the quantity of malondialdehyde (MDA) as an index of lipid peroxidation (Ohkawa et al. 1979). In *in vivo* experiments the individual mice were killed by cervical dislocation. Testes from 5 mice pretreated with $CdCl_2$ (2mg/kg, s.c.) for 24 hours and 5 mice pretreated with normal saline were promptly excised after decapitation, weighted, and chilled in ice-cold 0.9% NaCl. Another group of twenty mice were used for obtaining testicular tissue samples *in vitro* experiments. Tissue homogenates were prepared in a ratio of 1g of wet tissue of 9 ml of 1.15% KCl by using a ploytron homogenizer. The assay mixture contained 0.2 ml of the homogenates, 0.8 ml of 0.1 M phosphate buffer (PH 7.4), 3 ml of 1% phosphoric acid, 1 ml of 0.8% TBA-50% acetic acid solution and varying concentrations (0.1, 1.0, 10.0mM) of $CdCl_2$. The mixture was heated in a boiling water bath for 45 min and cooled in water. Then, the reaction products of TBA were extrated with 4 ml of n-butanol and were centrifuged (2,000 rpm) for 10 min. The absorbance of n-butanol solution at 532 nm was measured with UV-VIS spectrophotometer (Shimadzu) using 1,1,3,3-tetramethoxypropane as the standard, and expressed as nmol MDA formed.

HPLC Assay of DHBAs

High performance liquid chromatography (HPLC) system (Waters Associate Division of Millipore, Milford, MA) consisted of a Waters 501 HPLC pump, Water U6K universal liquid chromatography injector, an electrochemical detector model 460, equipped with a glassy carbon working electrode and auxiliary electrode and an Ag/AgCl reference electrode. The HPLC system was controlled by a Baseline 810 chromatography work station (Millipore, Milford, MA). The entire chromatographic system was run at ambient temperature and an oxidizing detector voltage was set at 0.8V. The column used was a Hibar RT, Lichrosorb-RP-18, 10um. 25cmx4mm (E. Merck). The mobile phase was prepared from distilled, deionized water and filtered before use with a 0.45μm filter (Millipore, Milford, MA). The mobile phase eluted at 0.8ml/min and was 0.03M citric acid and 0.03M acetic acid. The pH of the mobile phase was adjusted to pH 3.0 by saturated NaOH, followed by titration with saturated CH3COONa to a final pH of 3.6.

The individual mice within the control and treatment groups were given sodium salicylate 100mg/Kg, 30 min prior to killing by cervical dislocation. The testes were removed rapidly, weighed and placed in ice-cooled 10% TCA (1.5ml/200mg tissue). The testes were homogenized in a polytron homogenizer (Polytron model # PT 10/35, Brinkman Instruments, Switzerland) for 20 seconds. The homogenate was centrifugated at 2,000 RPM at room temperature for 10 minutes. The supernatants were filtered with a 0.22μm filter (Millipore, Milford, MA). 50μl aliquots were injected into the HPLC with electrochemical detector.

Using different concentrations of 2,5-DHBA and 2,3-DHBA added to the blank tissues and same extraction procedure, the amount of DHBAs present in the tissue extracts was determined by comparison of LCED peak area obtained on the extract to that of a standard curve obtained by diluting DHBAs in 10% TCA.

Statistical Analysis of the Data:

Student's t-test was used to determine the differences in Na^+, K^+-ATPase activities, MDA levels, GSH content, GSH/GSSG ratio and DHBAs of testes between control and various treatment groups. The results were considered significant at $P<0.05$.

RESULTS

Body and testes weight

The effects on body, testes weight and testes to body weight ratio of $CdCl_2$ are presented in Table 1. There was an apparent increase in testes weight at 12 hours and 24 hours followed by a significant reduction on day 5 through day 12 of treatment (control group = 234.0 ± 11.15mg Vs $CdCl_2$ day 5 group = 183.2 ± 12.44mg). There are some changes in testes to body weight ratio. However, there was no significant change in body weight.

Effects of $CdCl_2$ on in vivo Testicular Na^+, K^+-ATPase Activity

Subcutaneous administration of CdCl2 produced a dose-dependent inhibition of testicular microsomal Na^+, K^+-ATPase. Although the inhibitory effects of $CdCl_2$ at 0.5mg and 2mg/kg were minimal i.e. 6 and 12% respectively, there was a near 90% reduction in the enzyme activity at 2mg/kg/day for 2 days (Fig. 1).

Effects of $CdCl_2$ on in vitro Testicular Na^+, K^+ and Mg^{2+}-ATPase Activities

$CdCl_2$ produced a concentration-dependant inhibition of both Na^+, K^+ and Mg^{2+}-activated ATPase activities in testes (Fig. 2). The IC50 on Na^+, K^+-ATPase and Mg^{2+} activated ATPase

activities were 90 and 140μM respectively over 5 minutes preincubation.

Effects of CdCl$_2$ on Testicular GSH content and GSH/GSSG ratio

Subcutaneous administration of CdCl$_2$ produced a cumulative reduction in testicular GSH contents with significant effects on day 3 to day 12 (control group = 644.53±61.56μg/100mg Vs CdCl$_2$ day 3 group = 29.36±12.55μg/100mg). The ratio of GSH to GSSG was also reduced significantly with CdCl$_2$ treatment (Table 2).

Effects of CdCl$_2$ on Testicular Lipid Peroxidation

2mg/kg CdCl$_2$ (s.c.) increased MDA level in the testes of mice (Fig. 3). CdCl$_2$ also produced a concentration-dependent increase MDA *in vitro* (Fig. 4).

Effects of CdCl$_2$ on Testicular Hydroxyl Free Radicals Generation

Levels of 2,5-DHBA and 2,3-DHBA indicative of hydroxyl free radicals generation in the testes following CdCl$_2$ treatment are presented in Table 3. CdCl$_2$ induced a significant approximately of 3 to 4 fold increase in 2,5 DHBA and 2 to 7 fold increase in 2,3-DHBA respectively compared to that of control group. The ratio of 2,5-DHBA to 2,3-DHBA appeared to increase in CdCl$_2$ treated animals on 12 and 24 hours, day 5 and day 7 compared to that of control groups.

Effects of vitamin E Pretreatment on CdCl$_2$-induced Changes in Testicular Hydroxyl Free Radical Generation

Subcutaneous administration of CdCl$_2$ at 1mg/kg/day for 7 days produced a significant increase in testicular 2,5-DHBA and 2,3-DHBA in mice. Intramuscular administration of

vitamin E at 20mg/kg/day for 7 days 30 minutes prior to $CdCl_2$ administration produced a significant reduction in both 2,5-DHBA and 2,3-DHBA (Fig. 5 and 6).

DISCUSSION

The data presented in this study indicate that frequent administration of $CdCl_2$ at 1.0mg/Kg/day(s.c.) for a period of 12 days produced a cumulative effects on mouse testes without a significant alteration in body weight. Subcutaneous administration of $CdCl_2$ at this dose produced an increase in testicular weight within 12 hours followed by a time dependant reduction in testicular weight and in the weight ratio of body to testicular weight. It has been repeatedly shown that the testes are one of the most sensitive tissues to the acute toxic and chronic carcinogenic effects of cadmium (Waalkes and Oberdoster 1990). The hemorrhage, edema, necrosis and destruction of seminiferous tubules have been reported in previous studies (Gunn et al. 1968). The significant increase in testicular weight observed within 12 hours of $CdCl_2$ treatment at this dose could be due to damage to the capillary endothelial cells lining and subsequent increased permeability leading to testicular edema. The dramatic reduction in testicular weight and grayish-white color at 24 hours to 12th day may possibly due to $CdCl_2$ induced time dependent necrotic or degenerative changes.

We have shown for the first time that $CdCl_2$ produced a dose-related inhibition of testicular Na^+, K^+-ATPase both *in vivo* and *in vitro*. This is consistent with the results of numerous reports that the cadmium is a potent inhibitor of this membrane bound ATPase in variety of mammalian including human tissues (Samarawickrama 1979). ATPase, an integral part of the cell membrane plays an important role in the active transport of Na^+ and K^+ across cell membrane. It is the primary driving force for other membrane associated transport systems

or channels (Ca^{2+} channels, Na^+/Ca^{2+} and Na^+/H^+ antiporters and Na^+-dependent transporters for phosphate, glucose and amino acids). Thus Na^+, K^+-ATPase plays a vital role of a major communication system linking the extracellular signals to the intracellular medium, not only of neural tissues but also of non-neural tissues. The inhibition of these vital enzymes by cadmium could be damaging to the testicular cells leading to an earlier stage of edema followed by later stage of degeneration and necrosis. However, although most of the toxic metals including cadmium are known to bind with sulfhydryl groups of both α and β- subunits of Na^+, K^+-ATPase, the exact manner by which the cadmium brings about the inhibitory effects on Na^+, K^+-ATPase varies from species to species and perhaps also from organ to organ (Kinne-Saffran et al. 1993).

The results of the present study indicate that subcutaneous administration of $CdCl_2$ 1mg/Kg/day intermittently over a period of 12 days produced a significant reduction in mouse testicular GSH content and GSH/GSSG ratio. It has been established that the intracellular GSH plays a vital role in numerous cellular functions including detoxification of both exogenous and endogenous compounds. (Meister and Anderson 1983; Larsson et al. 1983; Singhal et al. 1987). GSH has been shown to form complexes with several heavy metals including cadmium and reduce their injurious or toxic effects (Singhal et al. 1987). It has been further demonstrated that various manipulations leading to depletion of intracellular GSH increased the toxic or lethal effects of various metals including cadmium in rodents (Singhal et al. 1987). Reduction of GSH in mouse testes is consistent with previous reports of cadmium induced alterations of the activities of enzymes such as glutathione peroxidase, glutathione reductase and catalase (Koizumi et al. 1992).

We have demonstrated for the first time that subcutaneous administration of $CdCl_2$ at 1mg/Kg/day produced a 3-4 fold increase in 2,5-DHBA and 2-7 fold in 2,3-DHBA in mouse

testicular tissue. Using salicylate as a trapping agent for hydroxyl radicals and quantitation by HPLC in combination with electrochemical detection (LCED), a significant increase in 2,5-DHBA and 2,3-DHBA as indices of highly reactive molecules have been shown in various target tissues subjected to toxic or ischemic insult (Floyd et al. 1984). The results of this study are consistent with numerous previous reports that the cadmium exerts its direct toxic effects on testicular vascular endothelium which could lead to ischemia, hypoxia and lipid peroxidation followed by generation of highly reactive hydroxyl free radicals in the testicular tissues (Gunn and Gould 1975; Aoki and Hoffer 1978; Koizumi et al. 1992). Thus, our own data provides direct evidence for the involvement of these free radicals in $CdCl_2$ induced acute testicular toxicity in mice.

It has been well established that vitamin E, one of the most commonly used antioxidant nutrients is very effective in preventing injurious effects produced by various toxicants and ischemic/reperfunsion insult (Liebler 1993). There is accumulating evidence that vitamin E is a versatile scavenger of highly reactive oxygen oxidation metabolites (Min et al. 1992). Thus, vitamin E alone or in combination with vitamins C, A, carotenoids and various phenolic compounds are believed to play a vital role in defending against tissue oxidative injuring caused by various toxicants and carcinogens (Liebler 1993; Pleasants et al. 1993). Our results presented in this study indicate that pretreatment with vitamin E produced a significant reduction in $CdCl_2$-induced increase in the formation of both 2,5 and 2,3 DHBA in mouse testicular tissue. These findings provide direct evidence that vitamin E could be effective in partially preventing $CdCl_2$ induced toxicity via a reduction in lipid peroxidation of testicular tissues and free radicals formation.

In conclusion, 1). 2,5-DHBA and 2,3-DHBA as indices of hydroxyl free radicals significantly increased in mouse testes after $CdCl_2$ treatment for 5 days, 2). Both content of

GSH and ratio of GSH/GSSG in testes decreased following $CdCl_2$ exposure, 3). $CdCl_2$ produced a concentration-dependent reduction in testicular microsomal Na^+,K^+-ATPase and increase in lipid peroxidation both *in vitro* and *in vivo*, 4).Pretreatment with vitamin E produced a significant reduction in $CdCl_2$-induced increase in testicular hydroxyl free radicals generation, and 5). an increase in hydroxyl free radicals generation and lipid peroxidation accompanied by a concomitant reduction in both GSH content and GSH/GSSG ratio and inhibition of microsomal Na^+,K^+-ATPase activity could play a vital role in the pathogenesis of $CdCl_2$-induced testicular toxicity.

REFERENCES

Akera T. and Brody T.M. (1969) The effects of ouabain on sodium-and potassium-activated adenosine triphosphatase from the hearts of several mammalian species. J Pharmacol Exp Ther 170:17-26

Ali M.M., Shukla G.S., Srivastava R.S., Mathur N. and Chandra S.V. (1993) Effects of vitamin E on cadmium-induced locomotor dysfunctions in rats. Vet Hum Toxicol 35:109-111

Ames B.N. (1983) Dietary carcinogens and anticarcinogens, oxygen radicals and degenerative diseases. Science 221:1256-1264

Aoki A. and Hoffer A.P. (1978) Reexamination of the lesions in rat testis caused by cadmium. Biol Reprod 18:579-591

Borel J.P. (1983) Radicals libries oxygenes, tissue conjontifet inflammation (1983) Pathol Biol 31:8-10

Broekhuysen J., Clinet M. and Delisee C. (1972) Action of amiodarone on guinea pig heart sodium and potassium activated adenosine triphosphatase, comparison with ouabain. Biochem Pharmacol 21:2951-2960

Bus J.S., Cagon S.Z., Olgaard M. and Gibson J.E. (1976) A mechanism of paraquat toxicity in mice and rats. Toxicol Appl Pharmacol 35:501-513

Chen C.L., Sangiah S., Patterson E. and Berlin K.D. (1992) Effects of BRB-1-28, a novel antiarrhythmic agent, and its derivatives on cardiac Na^+,K^+-ATPase, $Mg2+$-ATPase activities and contractile force. Res Comm Chem Path Pharm 78:3-16

Ewing D. (1983) Synergistic damage from H_2O_2 and OH^- radicals in irradiated cells. Radiation Res 94:171-189

Floyd R.A., Watson J.J. and Wong P.K. (1984) Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and salicylate hydroxylation products. J Biochem Biophys Methods 10:221-235

Floyd R.A., Henderson R., Watson J.J. and Wong P.K. (1986) Use of salicylate with high pressure liquid chromatography and electrochemical detection (LCED) as a sensitive measure of hydroxyl free radicals. J Free Radic Biol Med 2:13-18

Gabor S., Anca Z. and Bordas E. (1978) Cadmium-induced lipid peroxidation in kidney and testes. Rev Roum Biochem 15:113-117

Gunn S.A., Could T.C. and Anderson W.A.D. (1966) Selectivity of organ response to cadmium injury and various protective measure. Proc Soc Exp Med 122:1036-1039

Gunn S.A., Gould T.C. and Anderson W.A.D. (1968) Mechanism of zinc, cysteine and

selenium protection against cadmium-induced vascular injury to mouse testis. J Reprod Fert 15:65-70

Gunn S.A. and Could T.C. (1975) Vasculature of the testis and adnexa. In: Hamilton DW, Greep RO, Williams and Wilkins, Baltimore. pp. 117-142

Harman D. (1981) The aging process. Proc Natl Acad Sci 78:7124-7128

Heinrich U. (1988) Environmental toxin series 2: Cadmium. Stoeppler M and Piscator M, Springer-Verlag, FRG. pp. 14-24

Kinne-Saffran E., Hulseweh M., Pfaff C.H. and Kinne R.K.H. (1993) Inhibition of Na, K-ATPase by cadmium: Different mechanisms in different species. Toxicol Appl Pharmcol 121:22-29

Koizumi T., LI Z.G. and Tatsumoto H. (1992) DNA damaging acting of cadmium in leydig cells. a target cell population for cadmium carcinogenesis in rat testis. Toxicol Lett 63:211-220

Larsson A., Orrenius S. and Holmgren A. (1983) Function of glutathione: biochemical, physiological, and toxicological. Fifth Karolinska Institute Nobel Conference, New York, Raven.

Liebler D.C. (1993) The role of metabolism in the antioxidant function of vitamin E. Critical Rev toxicol 23:147-169

Meister A. and Anderson M.E. (1983) Glutathione. Annu Rev Biochem 52:711-760

Min K.S., Terano Y., Onosaka S. and Tanaka K. (1992) Induction of metallothionein synthesis by menadione or CCl4 is independent of free radical production. Toxicol Appl Pharmacol 113:74-79

Nohl H. and Jordan W. (1983) OH-generation by adriamycin semiquinone and H_2O_2; an explanation of the cardiotoxicity of anthracycline antibiotics. Biochem Biophys Res Commun 114:197-205

Ohkawa H., Ohishi N. and Yagi K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analyt Biochem 95:351-358

Pleasants E.W., Waslien C. and Naughton B.A. (1993) Dietary modulation of the symptoms of cadmium toxicity in rats: Effects of vitamin A,C,D hormone, and fluoride. Nutri Res 13:839-850

Preston A.M. (1991) Cigarette smoking-nutritional implications. Prog Food Nutr Sci 15:183-271

Ragan H.A. and Mast T.J. (1990) Reviews of environmental contamination and toxicology. Ware EW, Springer-Verlag, New York. pp. 1-22

Rajanwa B., Chapatwala K.D., Vaishnav D.D. and Dasaiah D. (1981) Changes in ATPase activities in tissues of rats fed on cadmium. J Environ 2:1-9

Samarawickrama G.P. (1979) The chemistry, biochemistry and biology of cadmium. Webb M, Elsevier/North-Holland Biomedical press, New York. pp. 341-421

Shen Y. and Sangiah S. (1994) Influence of cadmium on ketamine-induced anesthesia and brain microsomal Na^+, K^+-ATPase in mice. Bull Environ Contamin Toxicol 53:591-597

Shukla G.S., Hussain T. and Chandra S.V. (1987) Possible role of regional superoxide dismutase activity and lipid peroxides levels in cadmium neurotoxicity: in vivo and in vitro studies in growing rats. Life Sci 41:2215-2221

Singhal R.K., Anderson M.E. and Meister A. (1987) Glutathione, a first line of defense against cadmium toxicity. FASEB J 1:220-223

Slaga T.J. (1983) Overview of tumor promotion in animals. Environ Health Perspect 50:3-14

Smith P.K., Krohn R.I., Hermanson G.T. and Mallia A.K. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76-85

Tallarida R.J. and Murray R.B. (1986) Manual of Pharmacologic Calculations with Computer Programs. Tallarida RJ, Springer-Verlag, New York. pp. 131-134

Teare J.P., Punchard N.A. and poewll J.J. (1993) Automated spectrophotometric method for determining oxidized and reduced glutathione in liver. Clin Chem 39:686-689

Waalkes M.P. and Oberdoster G. (1990) Cadmium carcinogenesis. Foulkes ED, CRC Press, Boca Raton. pp. 129-158

Wahba Z.Z., Hernandez L., Issaq H.J. and Waalkes M.P. (1990) Involvement of sulfhydryl metabolism in tolerance to cadmium in testicular cells. Toxicol Appl Pharmacol 104:157-166

Yusa T., Crapo J.D. and Freeman B.A. (1984) Hyperoxia enhances lung and liver nuclear superoxide generation. Biochim Biophys Acta 798:167-174

Table 1. Effects of $CdCl_2$ (1mg/kg/day, s.c.) on body and testes weight in mice after different day treatment.

group	N	testes weight(mg)	body weight(g)	% of (TW/BW)[a]
control	11	234.0+11.15	32.83+0.93	0.71
Cd 12h	6	282.2+15.27	30.72+0.47	0.92
Cd 1day	10	258.6+14.94	31.62+0.85	0.82
Cd 3day	10	215.2+14.33	31.44+0.43	0.68
Cd 5day	10	183.2+12.44*	32.54+1.23	0.56
Cd 7day	10	126.2+8.96*	30.45+1.63	0.42
Cd 9day	10	132.6+15.72*	30.98+1.04	0.43
Cd 12day	10	147.3+11.15*	33.20+1.39	0.43

[a], testes weight/body weight.

*, signifcantly different from control group at P<0.05.

Table 2. The contents of GSH and GSSG in the testes of control and $CdCl_2$ (1mg/kg/day, s.c.) treated mice.

group	N	GSH(μg/100mg)	GSSG(μg/100mg)	GSH/GSSG(ratio)
control	5	644.53+61.56	110.27+6.22	5.84
Cd 1day	5	563.24+58.63	103.15+10.72	5.46
Cd 3day	5	279.36+12.55*	100.25+10.36	2.79
Cd 5day	5	272.38+25.24*	91.75+7.28	2.97
Cd 7day	5	256.61+43.11*	82.11+5.46*	3.12
Cd 9day	5	228.57+32.42*	82.94+6.49*	2.76
Cd 12day	5	244.43+25.67*	100.64+13.54	2.43

*, signifcantly different from control group at $P < 0.05$.

Table 3. The Levels of 2,5-DHBA and 2,3-DHBA in the testes of control and
CdCl$_2$(1mg/kg/day, s.c.) treated mice.

group	N	2,5-DHBA (nM/100mg)	2,3-DHBA (nM/100mg)	2,5-DHBA/2,3-DHBA (ratio)
control	6	324.22+8.34	37.79+4.05	8.58
Cd 12h	6	466.89÷65.39*	23.22+3.12*	20.11
Cd 1day	5	427.40+33.76*	26.68÷2.52	16.02
Cd 3day	5	352.84+38.91	46.39+5.94	7.61
Cd 5day	5	821.45+70.86*	69.21+8.72*	11.87
Cd 7day	5	990.37+127.90*	71.21+7.09*	13.91
Cd 9day	5	1092.25+161.14*	181.13+37.44*	6.03
Cd 12day	5	973.32+106.67*	269.48+28.25*	3.61

*, signifantly different from control group at $P < 0.05$.

FIGURE LEGENDS:

Fig. 1: Effects of $CdCl_2$ on *in vivo* testicular microsomal Na^+-ATPase in mice.

Fig. 2: Effects of $CdCl_2$ on *in vitro* testicular micorsomal ATPase in mice.

Fig. 3: Effects of $CdCl_2$ on *in vivo* testicular lipid peroxidation in mice.

Fig. 4: Effects of $CdCl_2$ on *in vitro* testicular lipid peroxidation in mice.

Fig. 5: The levels of 2,3-DHBA in the testes of control, cadmium chloride, and vitamin E plus cadmium chloride-treated mice. *$P<0.05$.

Fig. 6: The levels of 2,5-DHBA in the testes of control, cadmium chloride and vitamin E plus cadmium treated mice. *$P<0.05$.
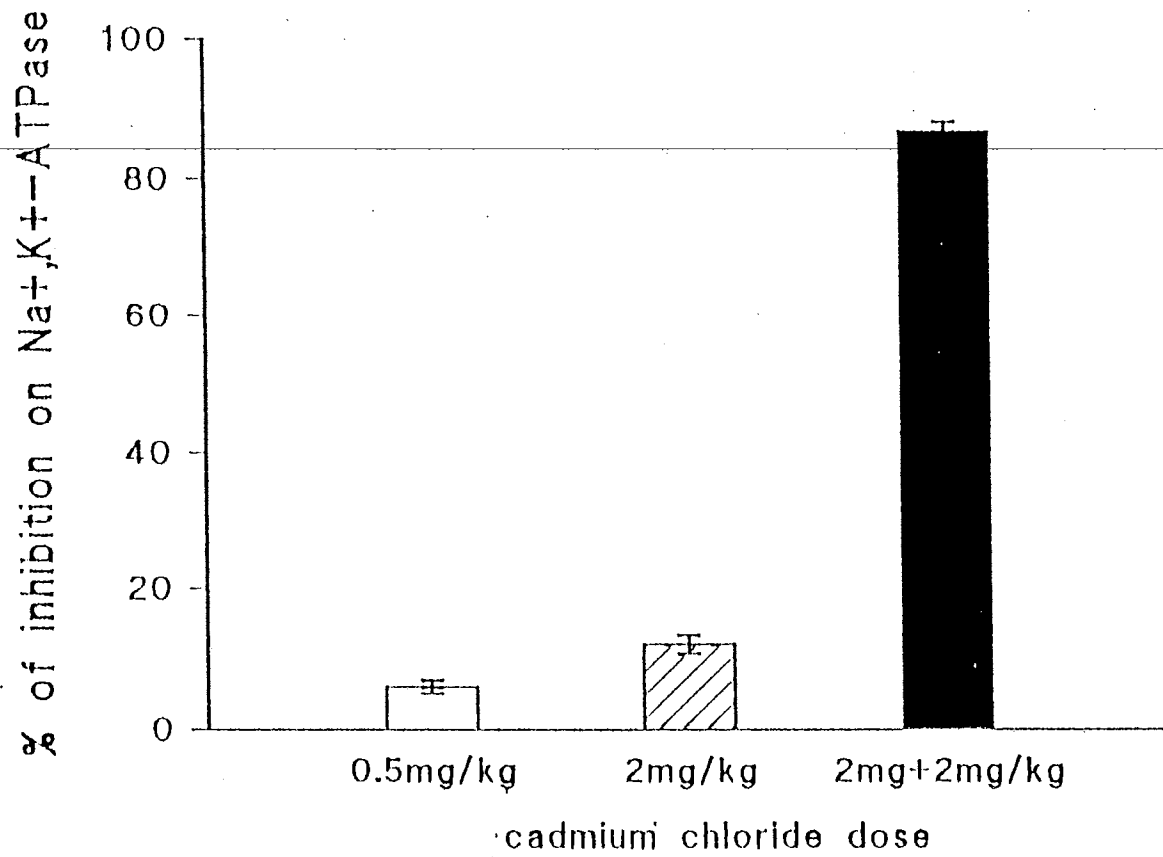
Fig. 1. Effects of cadmium chloride on in vivo testicular
microsomal Na+,K+—ATPase in mice.

Fig. 1
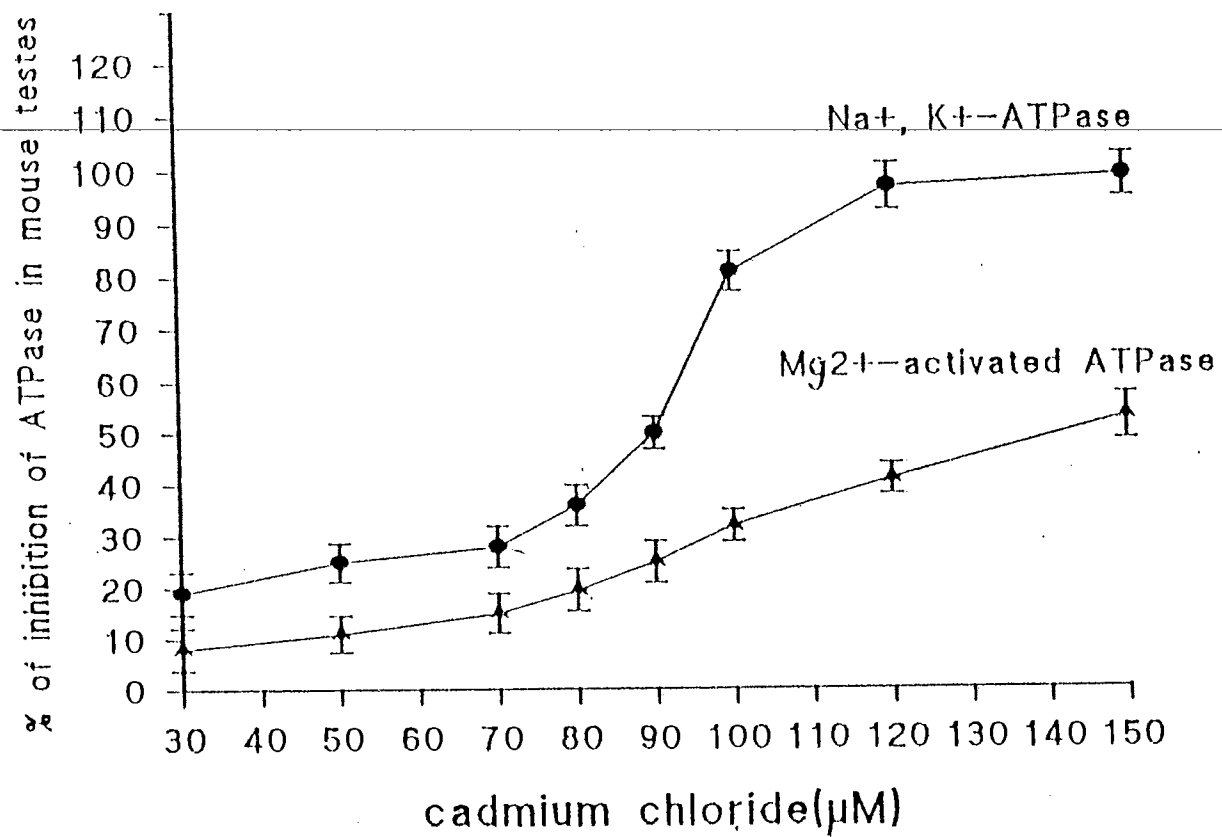
Fig. 2. Effects of cadmium chloride on in vitro testicular microsomal ATPase.

Fig. 2
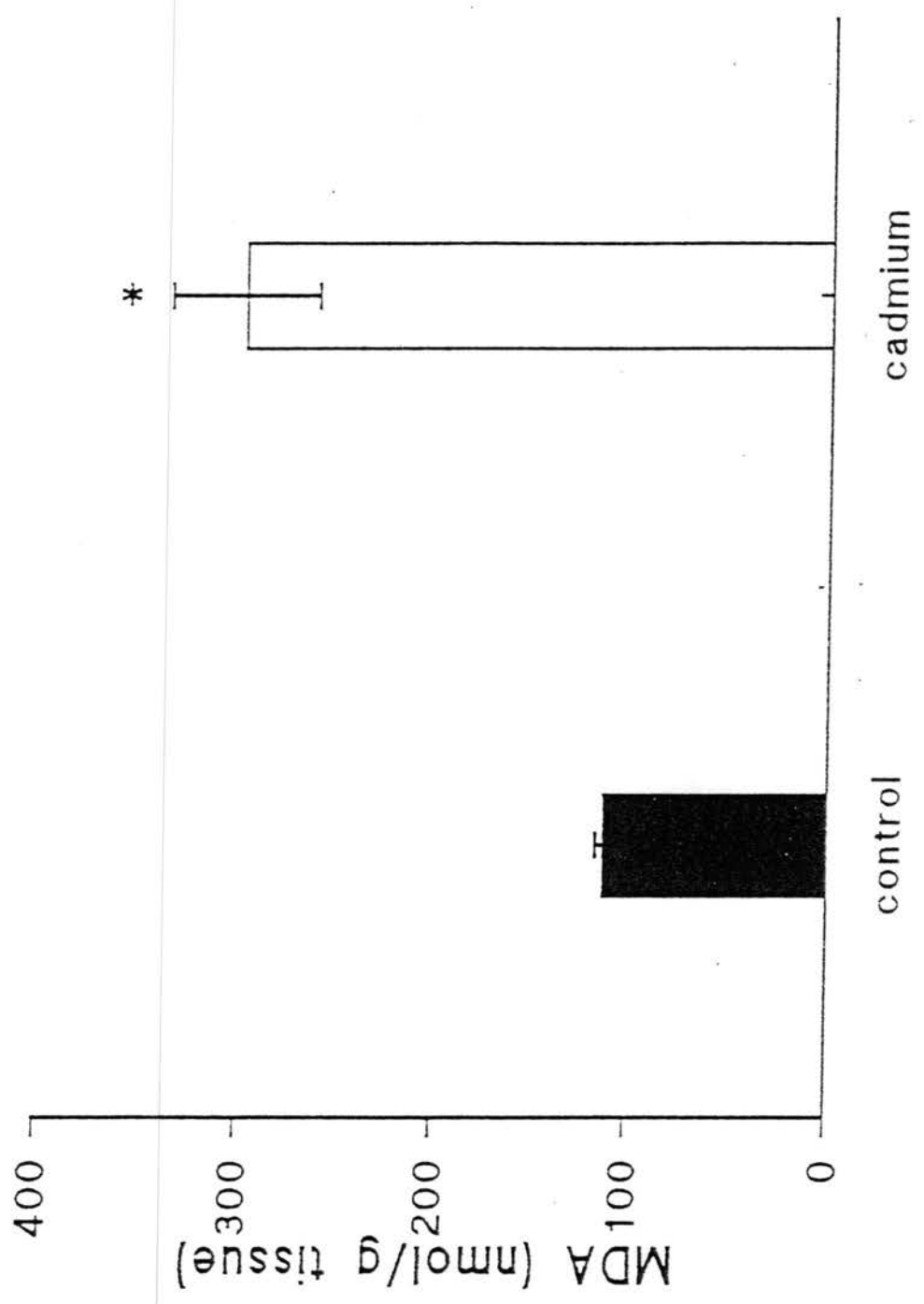
Fig. 3
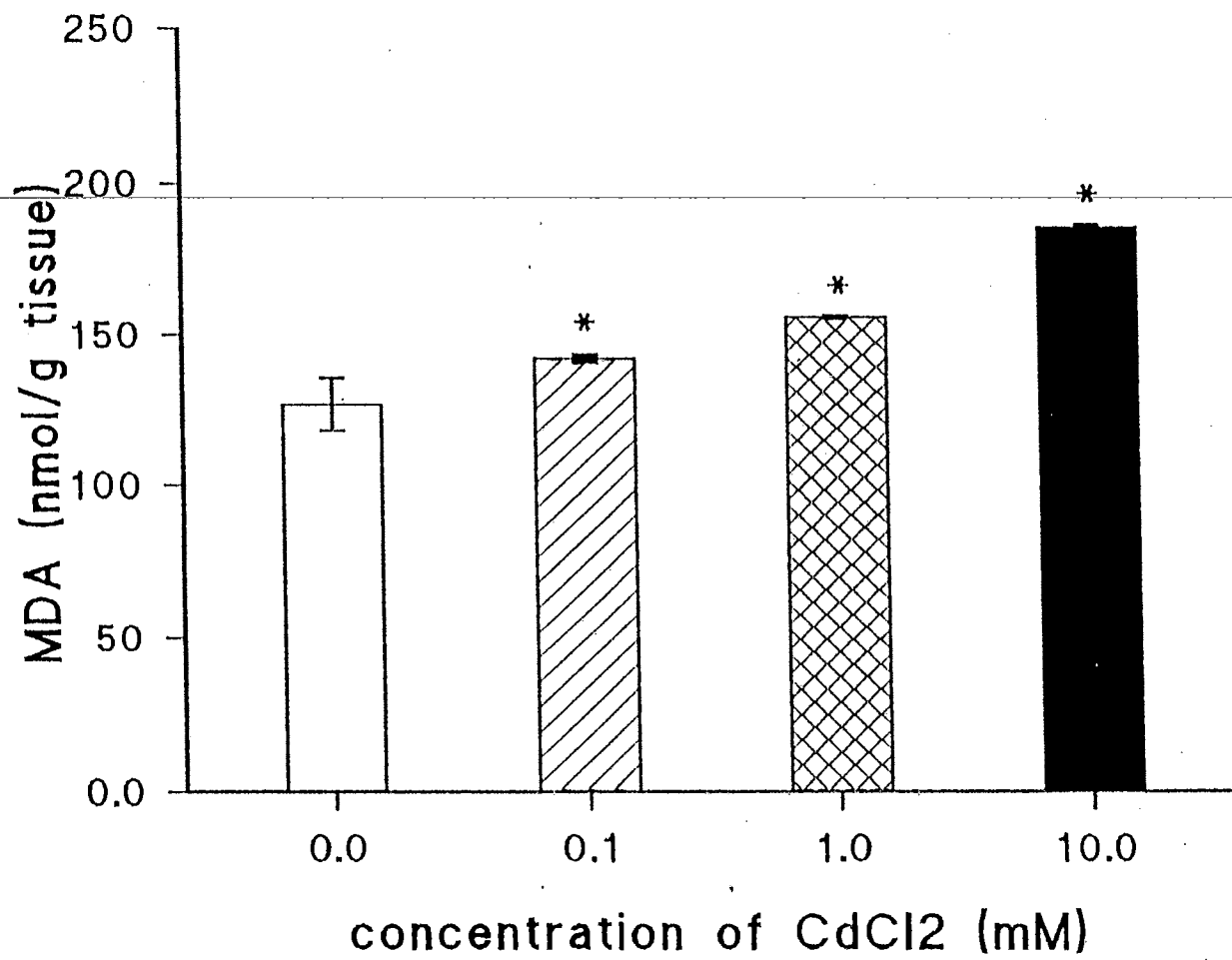
Fig. 4
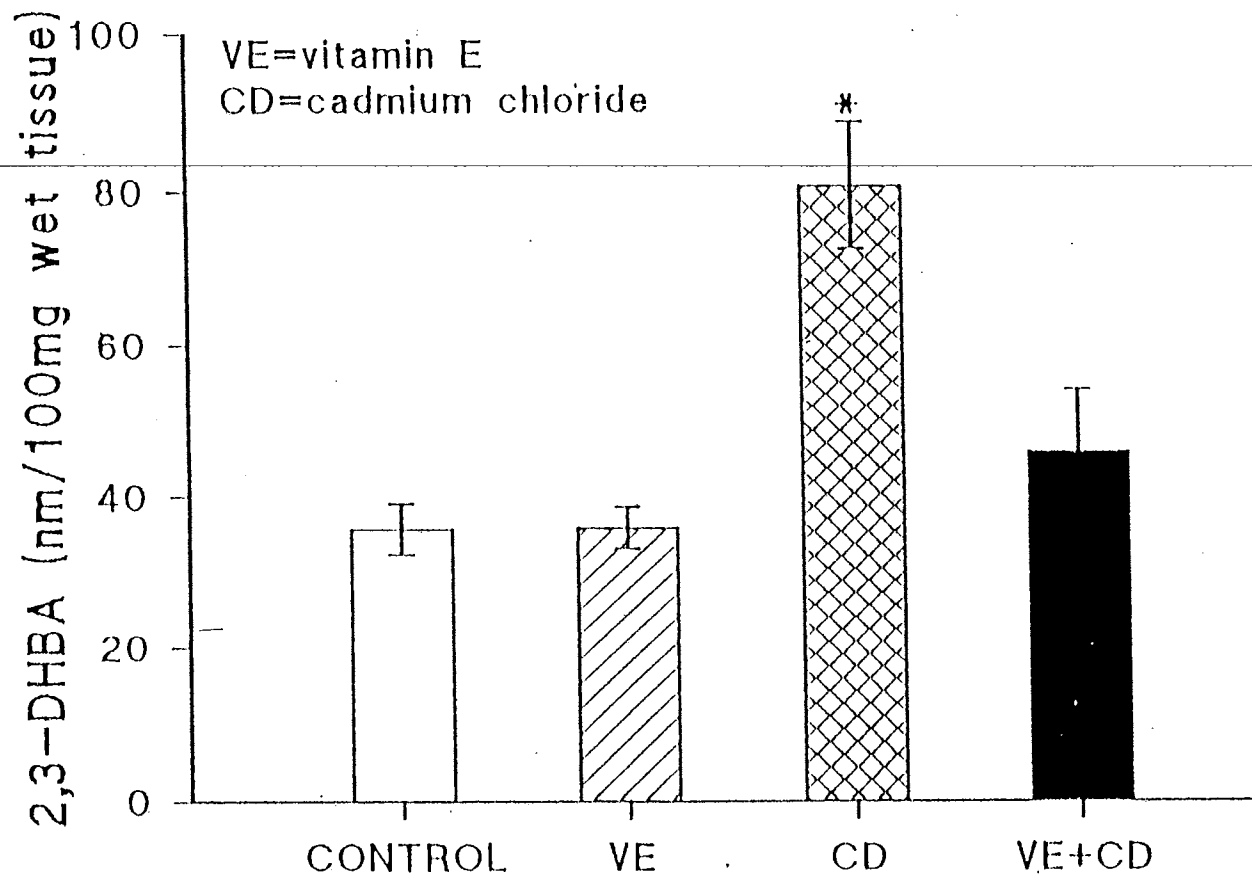
Fig. 5 . The level of 2,3-DHBA in the testes of control, cadmium chloride and vitamin E plus cadmium chloride treated mice. * P<0.05.

Fig. 5
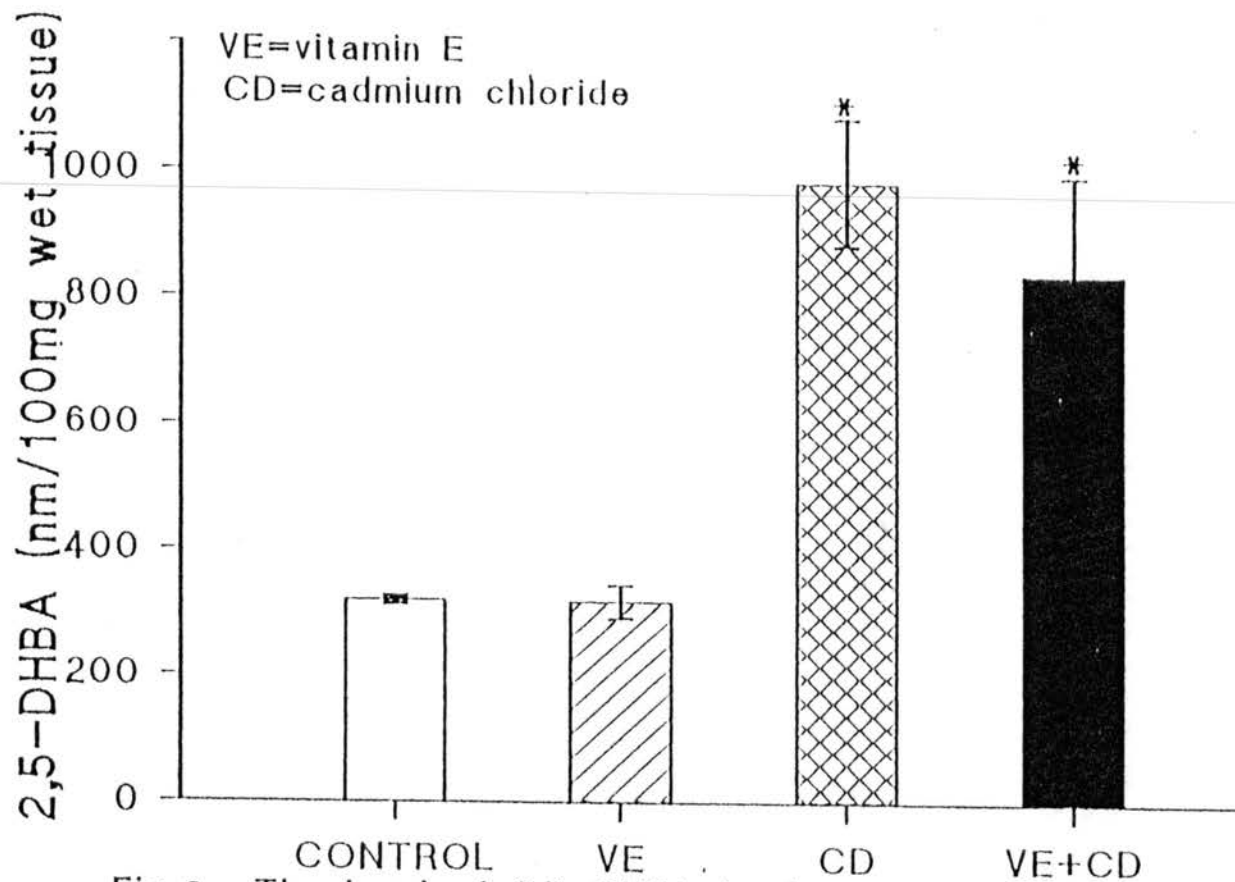
Fig.6 . The level of 2,5-DHBA in the testes of control, cadmium chloride and vitamin E plus cadmium chloride treated mice. * P<0.05.

Fig. 6

CHAPTER IV

RESPONSE SURFACE ANALYSIS OF THE INFLUENCES OF DIETARY SULFUR CONTAINING AMINO ACIDS ON CADMIUM INDUCED BIOCHEMICAL CHANGES IN RATS

ABSTRACT

Response surface regression analysis was used to study the influences of dietary levels of methionine(Met) and cystine(Cys) on the effects of Cd-induced biochemical changes in rats. It was used to evaluate liver and kidney metallothionein (MT), brain, heart, liver and testis glutathione(GSH), brain, liver and testis lipid peroxidation and intestinal mucosal ATPase activities. The blood chemistry data also were included in this study. Because the dietary components were not statistically independent, they were studied in combination of two variables (Met and Cys). The regression coefficients and estimated parameters of these response surfaces and three dimensional plots were developed for each of the biochemical markers to help determine the influences of dietary Met and Cys either alone and in combination on Cd toxicity. Rats were randomly assigned to one of nine experimental diets containing variable concentrations of Met (0.00 to 0.80g/100g diet) and Cys(0.00 to 1.5g/100g diet). The individual rats within each experimental group received $CdCl_2$ (100ppm) in drinking water daily for 10 weeks. After 10 weeks the organs and blood were collected for biochemical analysis. Dietary Met had a significant linear effect on liver MT, Met and Cys had a significant interactive effect on brain GSH. Cys had a significant quadratic effect on testis

GSH. and Met had a significant effect on intestinal Mg^{2+}-ATPase activity. These results are discussed with respect to the impact of dietary sulfur containing amino acids on liver, kidney and testicular tissue GSH and MT in Cd-induced toxicity in rats.

INTRODUCTION

Cd is a rare metallic element and it is present in almost all types of food. Shellfish, wheat and rice accumulate very high amounts of Cd. Occupational and environmental pollutants are the main sources of Cd exposure. Cd has a very long biological half-life. Exposure to Cd causes anemia, hypertension, hepatic, renal, pulmonary and cardiovascular disorders. It is considered to be a possible mutagen, teratogen and carcinogen (Friberg and Elinder 1992).

Both glutathione (GSH) and metallothionein (MT) in the target organ cells have been shown to play a crucial role in detoxification of Cd (Sies and Wendel 1978; Klaassen et al. 1985). GSH is a nonprotein thiol which is involved in cellular protective mechanisms that modulate responses to toxic agents. The structure of GSH was established as r-glutamylcysteinylglycine (r-Glu-Cys-Gly) through chemical analysis, acid-base titration, degradation and synthesis (Kosower 1978). GSH protects against toxic damage in the cells. It forms complexes with heavy metals and protects cells against metal toxicity. This tripeptide provides a first line of defense against Cd before induction of MT synthesis occurs (Singhal et al. 1987). Rats that were pretreated with agents which depleted the GSH concentration in liver cells were much more susceptible to Cd administered intravenously at 1.3 or 2.0 mg/kg (Dudley and Klaassen 1984). GSH-depleted animals had high mortality and showed profound morphological and biochemical signs of liver toxicity. In control rats, given Cd alone, there

were no deaths and only minor morphological and biochemical changes.

Mammalian MT contains 61 amino acids, of which 20 are cysteine. It is an unique low molecular weight intracellular metalloprotein and contains no aromatic amino acids or histidine (Kagi and Vallee 1960). The low molecular weight protein contains no free-groups and disulfide bridges *in vivo* and always binds with metals (Elinder and Nordberg 1985). Its synthesis is induced by a number of metals as well as various other environmental factors. MT plays a major role in the metabolism and toxicity of Cd. It sequesters Cd in the cells and thereby acts as a detoxifying agent. MT and GSH constitute two major intracellular cysteine pools in the liver. It has been previously demonstrated that when rats were fed on a soya-bean-protein diet, low in sulfur containing amino acids, the hepatic GSH levels of rats were decreased (Taniguchi and Cherian 1990). It is also reported that when the rats were fed on diets lacking cysteine for 8 days, the hepatic GSH levels were decreased approximately 75% (Sendelbach et al. 1990).

The toxic effects of a single intraperitoneal dose of cadmium in rats have been shown to be attenuated by prior administration of a single dose of cysteine, a sulfur containing amino acid and an essential precursor of GSH and MT (Dudley and Klaassen 1984). Sulfur containing amino acids such as cysteine, cystine and methionine play an important role in Cd toxicity. Cysteine can reverse the toxic effects of Cd. Cd, administered subcutaneously, causes complete necrosis of the testis in mice. This effect can be prevented by prior administration of cysteine (Gunn et al. 1966). Cysteine also has been shown to change the transportation and distribution of Cd and reverse testicular damage in mice (Gunn et al. 1968). The reversal of Cd toxicity with cysteine could possibly be attributed to its thiol groups (Bousquet 1979). Pretreatment with cysteine, which increased the liver GSH levels, markedly decreased the

liver toxicity of 3.9 mg/kg Cd given intravenously (Elinder 1986).

Dietary sulfur containing amino acids are released into the blood by the splanchnic organs and removed by the liver. Cystine occurs normally in our food sources. Cysteine and cystine are in fact easily interconvertible in the body. Other sulfur containing amino acids also are related to cysteine metabolism. The amino group and carbon skeleton of cysteine can be provided by serine, whereas the sulfur is derived from methionine. More than half of the sulfur containing amino acids are utilized for the biosynthesis of GSH (Garcia and Stipanuk 1992). Studies concerning the influence of dietary sulfur containing amino acids such as methionine and cystine on Cd toxicity are nonexistent at present.

Response surface regression analysis has been used as an analytical tool in sensory evaluation (Covanni 1983; Schutz 1983), in digestibility studies (George et al. 1980) and in determination of a balanced diet for maximal body energy gain and body protein gain (Toyomizu et al. 1985). This procedure allows the use of two or more independent variables to determine maximum and minimum responses of the dependent variables. However, no research has been conducted to investigate the interactive effects of sulfur containing amino acids on biochemical changes of vital organs due to Cd toxicity.

The present studies were designed to examine the influence of dietary sulfur containing amino acids such as cystine and methionine either alone or in combination on Cd induced-biochemical changes in vital organs of rats, using response surface regression analysis.

MATERIALS AND METHODS

Chemicals and Regents

All the reagents used in this study were of analytical grade. $CdCl_2$, GSH, 5,5-Dithiobis-2-nitrobenzoic Acid(DTNB), β-Nicotinamide Adenine Dinucleotide Phosphate(NADPH), glutathione reductase(GR), picric acid, MT and all other chemicals used for the ATPase enzyme assays were obtained from Sigma Chemical Co. (St Louis, MO). Protein regent was purchased from Pierce Chemical Co. (Rockford, IN). All solutions used for these studies were prepared using Milli-Q (Millipore, Bedford, MA) double distilled water.

Animals

Thirty nine, 4 weeks old, mycoplasma and specific virus-antigen-free, male Sprague-Dawley (Sasco Labs., Omaha, NE) rats (120-140g) were used in this study. They were housed in individual stainless steel cages with wire mesh floors at constant temperature, humidity, and air circulation facility according to the Guide for Care and Use of Laboratory animals (NIH, 1985). They were kept on a 12 hr light-dark cycle. One week after arrival, the rats were randomly assigned to 9 different treatment groups.

Diets

Basal diets (Harlan Teklad, Madison, WI) with the following compositions (Table 1) were mixed with different levels of cystine and methionine in powder form. The diets were fed to the individual rats within the treatment groups.

TABLE 1
MODIFICATION OF DIET AIN-93M
(Harlan Teklad)

ingredients	g/kg
CASEIN	140.0
SUCROSE	100.0
CORN STARCH	467.296---467.458
MALTODEXTRIN[1]	155.0
SOYBEAN OIL	40.0
CELLULOSE[2](fiber)	50.0
MINERAL MIX, AIN-93M-MX	35.0
VITAMIN MIX, AIN-93-VX	10.0
CHOLINE BITARTRATE	2.5
TBHQ[3] (antioxidant)	0.008

[1]Dextrinized corn starch (hydrolysate, 90-94% tetrasaccharides and
   higher).
[2]Solka-Floc@, 200FCC.
[3]tert-butylhydroquinone.

Various levels of DL-Methionine(Met) and L-Cystine(Cys) were added to the basal

diet according to the experimental design, presented in Tab. 2.

Experimental Design

The experimental design is a central composite rotatable design with two x-variables.

Following one week adaptation, with regular powder diet (containing 0.3% cystine in

basal diet), the individual rats within the treatment groups were fed on the experimental

diets plus 100ppm $CdCl_2$ in drinking water daily for 10 weeks. Body weight and general

conditions were recorded each week. Food and water consumptions were recorded every

two days, throughout the 10 week period. All of the rats from each group were killed by

decapitation with a guillotine following 24 hours starvation at the end of the 10 week

period.

TABLE 2

Central composite rotatable design and
corresponding variable settings

rats	x1	x2	Met(x1) (g/100g diet)	Cys(x2) (g/100g diet)
3	-1	-1	0.12	0.22
3	-1	1	0.12	1.28
3	1	-1	0.68	0.22
3	1	1	0.68	1.28
3	0	$-\sqrt{2}$	0.40	0.00
3	0	$\sqrt{2}$	0.40	1.50
3	$-\sqrt{2}$	0	0.00	0.75
3	$\sqrt{2}$	0	0.80	0.75
15	0	0	0.40	0.75

Statistical Analysis of the Data

A two-factor central composite rotatable response surface design was used in these experiments with the following equation:

$$y = b_0 + b_1 x1 + b_2 x2 + b_{11} x1^2 + b_{22} x2^2 + b_{12} x1 x2$$

The relationship between the measurements (biochemical changes), y, and the corresponding variable settings, x, is modeled. Here b_0 is the model intercept, x1 and x2 are the levels of Met and Cys in rat diet, b_1 and b_{11} are parameters associated with the effects of Met, b_2 and b_{22} are parameters associated with the effects of Cys, and b_{12} is the parameter associated with the interactions of Met and Cys. The estimated parameters were determined using the PROC RSREG procedure of the Statistical Analysis System (SAS). In all analyses, P value < 0.05 was considered significant. Three dimensional response surface contour plots were produced for each variable using the three dimensional graphics procedure (G3D).

The optimal stepwise regression equation was selected for each variable, using the

criteria that the last variable entered resulted in a significant improvement in the response surface regression analysis.

Experimental Procedures

Determination of MT in Liver and kidney

The levels of MT in liver and kidney were determined by HPLC method. Tissues were promptly excised and 1 g tissue sample was prepared by homogenizing the tissue in 5 ml NaH_2PO_4 buffer (pH 7.0) containing 10 mM ascorbic acid. The homogenates were centrifuged for 90 min at 31,000 rpm. After centrifugation, the supernatants were heated for 10 min at 60°C, followed by cooling in ice for 10 min. Then they were subjected to centrifugation to remove the heat-coagulated protein from the homogenate. The resulting supernatants were filtered through a 0.22 μm pore membrane (Millipore Co., Bedford, MA) prior to HPLC analysis for MT.

The HPLC system (Millipore, Milford, MA) consisted of a Waters 501 HPLC pump, Waters U6K universal liquid chromatography injector, and a Model 484 Tunable Absorbance Detector controlled by a Baseline 810 chromatography work station (Millpore, Milford, MA). A μBondapak C_{18} (3.9mmx300mm) stainless steel column (Waters, Millipore, MA) was used. The mobile phase used to elute MT from the reversed-phase column consisted of an equilibration buffer (buffer A) containing 10 mM NaH_2PO_4, pH 7.0, and an elution buffer (buffer B) that contained an organic modifier, 60% acetonitrile dissolved in buffer A. MT was eluted with a gradient formed by mixing an increasing amount of buffer B with buffer A over a specified period of time. The column was maintained at ambient temperature and eluted at a flow rate of 1.6 ml/min. The column effluent was monitored for Ultraviolet Absorbance

(UV) of MT at the detection wave length of 214 nm. Injections were 50 µl containing 50 µg of purified rabbit liver MT (Sigma Chemical Co., St Louis, MO) in buffer A as standard or 50 µl tissue supernatant. The linear gradient consisted of 0-10% buffer B from 0 to 5 min following by 10-25% buffer B from 5 to 25 min and 25-100% buffer B from 25 to 40 min. Following gradient elution of MT, the column was purged with 100% buffer B for 20 min and equilibrated with 100% buffer A for 10 min prior to injection of the next sample.

Determination of GSH in Brain, Heart, Liver and Testis

The levels of GSH in tissues were determined by using modified procedures previously reported (Teare et al. 1993). Standards were prepared in water containing picric acid such that the concentrations of picric acid in the final reaction mixture were the same as for the diluted biological sample supernates (0.35 mmol/L for GSH). NADPH (0.03 mmol/L) was dissolved in 0.125 mol/L sodium phosphate buffer (pH 7.5) containing 6.3 mmol/L sodium EDTA. DTNB (6.0 mmol/L) and GR solutions (1 U/100µl) were also prepared in this buffer. Blanks containing picric acid only were prepared.

After sacrificing the individual rats within each treatment group, brain, heart, liver and testes were removed rapidly, weighed and placed in ice-cooled 5 ml (43 mmol/L) of picric acid reagent per gram of tissue. The individual organs were homogenized with a polytron homogenizer (Polytron model # PT 10/35, Brinkman Instruments, Switzerland) for 50 seconds. The homogenates were centrifuged (5,000 rpm at 4°C for 20 min) and supernates from the individual tissue homogenates were divided into aliquots which were then stored at -70°C.

For GSH determination, 100 µl supernates were added to 9.9 ml of ice-cold water. 140

μl of NADPH reagent, 20 μl of DTNB reagent, and 20 μl of sample or standard were pipetted into the microplate and incubated at 30°C for 10 minutes. Then 20μl of GR was added, and the reaction was monitored immediately with readings taken every 9 seconds for 5 minutes. The reaction was monitored over the linear part of the curve.

Determination of Malondialdehyde (MDA) as an Index of Lipid Peroxidation in Brain, Liver and Testis

The thiobarbituric acid (TBA) assay was used to measure the amount of MDA as an index of lipid peroxidation (Ohkawa et al. 1979). Tissues were promptly excised, weighed, and chilled in ice-cold 0.9% NaCl. Tissue homogenates were prepared in a ratio of 1g of wet tissue in 9 ml of 1.15% KCl by using a homogenizer (Polyton model # PT 10/35, Brinkman Instruments, Switzerland). The assay mixture contained 0.2 ml of the individual tissue homogenates, 0.8 ml of 0.1 M phosphate buffer (PH 7.4), 3 ml of 1% phosphoric acid, and 1 ml of 0.8% TBA-50% acetic acid solution. The mixture was heated in a boiling water bath for 45 min and cooled in water. Then, the reaction products of TBA were extracted with 4 ml of n-butanol and were centrifuged (2,000 rpm) for 10 min. The absorbance of n-butanol solution at 532 nm was measured with a UV-1201 spectrophotometer(Shimadzu, Columbia, MD) using 1,1,3,3-tetramethoxypropane as the standard, and expressed as nmol of MDA formed.

Determination of Intestinal Mucosal ATPase Activity

Microsomal ATPase enzymes activities in the intestinal mucosa were determined following previously established procedures (Akera and Brody 1969; Chen et al. 1992; Shen and

Sangiah 1994). ATPase activity was measured by the method of Broekhuysen et al. (1972) with modifications as previously reported (Chen et al. 1992; Shen and Sangiah 1994). The reaction was initiated by adding ATP and the final concentration was 3.0 mM. The inorganic phosphate was determined spectrophotometrically at 660 nm and each assay was performed in duplicate. The Na^+, K^+-ATPase activity was calculated by subtracting the Mg^{2+}-activated ATPase activity from total ATPase activity. All results were corrected for blank values without enzyme. IC_{50} was calculated using computer software (Tallarida and Murray 1986).

Determination of Clinical Chemistry Profiles

The individual rats within the treatment groups were lightly anesthetized with methoxyflurane. Blood sample was drawn from each of the rat hearts and placed into a specific tube (Vacutainer, Becton Dickinson, Rutherford, NJ) for serum. Glucose, serum urea nitrogen, creatinine, total protein, albumin, calcium, phosphorus, cholesterol, alanine aminotransferase(ALT), aspartate aminotransferase(AST), alkaline phosphatase, sodium, potassium and chloride were measured using a Roche Cobas Mira (Roche Analytical Instruments, Nutley, NJ).

RESULTS

Effects on Body Weights, Weight Gain, Food and Water Consumption

Feeding the rats with the experimental diets containing different levels of Met and Cys for ten weeks produced no significant differences in their final weights and weight gain between treatment groups (data not presented). A control group fed on AIN-93M control

diet was not included in the central composite design. On comparing the control group with the other nine groups, there were no significant differences in final weights and weight gain. There were no significant differences in food and water consumption within the nine groups of the rats included in the central composite design. However, compared with the control group, both food and water consumption of animals receiving 100ppm $CdCl_2$ in drinking water daily for 10 weeks were found to be significantly($P<0.05$) decreased (data not presented).

Effects on MT

Mean liver and kidney MT levels for the rats fed on the experimental diets are shown in Tab. 3. Values for liver MT ranged from 31.36 to 88.74 nmol/g wet tissue. From the response surface regression equation it was observed that Met(x1) had a linear effect ($P<0.05$) on liver MT. From the three dimensional response surface plot(Fig. 1) it was observed that at a higher dietary Cys intake, MT increased as dietary Met increased. When the data were analyzed by using stepwise regression, the optimal regression equation was found to be y(liver MT)=51.51+37.27Met(x1)^2 ($P<0.05$).

The values for kidney MT ranged form 9.18 to 18.96 nmol/g wet tissue. In the kidney, neither Met(x1) nor Cys(x2) had significant effects on MT.

Effects on GSH

Mean brain, heart, liver and testis GSH contents in the rats fed on the experimental diets containing different levels of sulfur amino acids for 10 weeks are presented in Table 4. . Values for brain GSH content ranged from 49.89 to 136.03 μg/100mg wet tissue. From the

response surface regression equation, it was observed that Cys(x2) had a linear effect (P<0.05) and Met(x1) Cys(x2) had significant interactive effects (P<0.05) on brain GSH content(Fig. 2). When the data were analyzed by using stepwise regression, the optimal regression equation was y(brain GSH)=91.35-33.01Met(x1)+68.08Met(x1)Cys(x2) (P<0.05).

Values for testis GSH ranged from 151.25 to 370.25 µg/100mg wet tissue. From the response surface regression equation it was observed that Cys(x2) had a quadratic effect (P<0.05) on the testis GSH content (Fig. 3). When the data were analyzed by using stepwise regression, the optimal regression equation was found to be y(testis GSH) = 220.97 +275.02Cys(x2)-183.12Met(x1)^2-222.33Cys(x2)^2+145.42Met(x1)Cys(x2) (P<0.05). The critical values for the maximum point of testis GSH content were 0.337(g/100g diet) for Met and 0.733(g/100g diet) for Cys.

Values for heart GSH ranged from 193.96 to 232.81 µg/100mg wet tissue and values for liver GSH ranged from 334.90 to 790.71 µg/100mg wet tissue. Neither Met(x1) nor Cys(x2) had significant effects on GSH contents of heart or liver.

Effects on MDA levels (lipid peroxidation)

The mean brain, liver and testis MDA levels are presented in Table 5. Values for brain ranged from 114.35 to 149.58 nmol MDA/g wet tissue. Response surface analysis for brain MDA levels showed that the crossproduct regression was significant(P<0.05) for the combination of Met and Cys in the diet. From the response surface regression equation Met(x1) and Cys(x2) had significant interactive effects(Fig. 4). Using the stepwise regression equation, the optimal regression equation was found to be y(brain MDA level) = 161.48 - 61.88Met(x1) - 32.02Cys(x2) + 82.53Met(x1)Cys(x2) (P<0.05).

Values for liver MDA level ranged from 166.63 to 263.85nmol MDA/g wet tissue. From the response surface regression equation, it was observed that Cys(x2) had significant linear and quadratic effects (P<0.05) on liver lipid peroxidation (Fig. 5). Using stepwise regression equation, the optimal regression equation was y(liver MDA level) = 174.96 + 107.69Cys(x2) + 49.94Met(x1)^2 - 64.75Cys(x2)^2 (P<0.05).

Values for testis MDA level ranged from 100.34 to 129.77 nmol MDA/g wet tissue. From response surface regression equation, Cys(x2) had a significant quadratic effect (P<0.05) on the testis lipid peroxidation (Fig. 6). Using the stepwise regression, the most predictive model using the criteria that the last variables entered resulted in a significant improvement for testis lipid peroxidation was: y (testis MDA level)= 122.64 - 24.72Cys(x2) + 18.17Cys(x2)^2 (P<0.05). The critical values for the minimum point of the testis lipid peroxidation were 0.495(g/100g diet) for Met and 0.705(g/100g diet) for Cys.

Effects on ATPase activity

Mean intestinal mucosal total ATPase, Na$^+$,K$^+$-ATPase and Mg^{2+}-activated ATPase activities are presented in Table 6. The values for Mg^{2+}-ATPase ranged from 118.59 to 314.55 nM Pi/mg protein/min. From the response surface regression equation, Met(x1) had significant linear and quadratic effects (P<0.05) on intestinal mucosal Mg^{2+}-ATPase(Fig. 7). Using stepwise regression equation, the most predictive model using the criteria that the last variable entered resulted in a significant improvement for Mg^{2+}-ATPase was: y(Mg^{2+}-ATPase)=248.62-317.65Met(x1)+300.33Met(x1)^2 (P<0.05). On the intestinal mucosa the values for total ATPase ranged from 195.17 to 455.78 nM Pi/mg protein/min and for Na$^+$, K$^+$-ATPase ranged from 104.45 to 185.35 nM Pi/mg protein/min. For total ATPase and

Na^+,K^+-ATPase activities neither Met(x1) nor Cys(x2) had significant effects.

Effects on blood chemistry data

The analyses of blood chemistry data showed that Met(x1) had significant (P<0.05) quadratic effects on ALT(Fig. 8). Using stepwise regression equation, the optimal regression equation was: $y(ALT)=45.34 + 164.40 \ Met(x1)^2 +77.69Cys(x2)^2 -169.02Met(x1)Cys(x2)$ (P<0.05). The critical values for the minimum point of the ALT were 0.29(g/100g diet) for Met and 0.22(g/100g diet) for Cys.

From the response surface regression equation it was observed that Cys(x2) had significant quadratic effects (P<0.05) on AST(Fig. 9). Using stepwise regression equation, the optimal regression equation was: $y(AST)=184.98+123.52Cys(x2)^2$ (P<0.05). The critical values for the minimum point of AST were 0.35(g/100g diet) for Met and 0.47(g/100g diet) for Cys.

From the response surface regression equation, Met(x1) had significant linear and quadratic effects (P<0.05) on blood creatinine (Fig. 10). Using stepwise regression, the most predictive model using the criteria that the last variable entered resulted in significant improvement in the model for creatinine was: $y(creatinine)=0.60 +0.51Met(x1)-0.70Met(x1)^2$ (P<0.05). The critical values for the maximum point of blood creatinine were 0.36(g/100g diet) for Met and 0.71(g/100g diet) for Cys.

No significant response surface effects were found on other blood chemistry data.

DISCUSSION

The typical dietary intake of Cd in the United States has been estimated to be approximately 50μg/day for adults (Mahaffey et al. 1975). The uptake and retention of Cd and hence its toxicity may greatly depend on nutritional factors such as dietary contents of proteins or essential trace metals. Supplements of several essential nutrients in excess of requirements have been shown to reduce Cd toxicity in experimental animals. The type of dietary protein in purified diets was found to influence markedly the severity of Cd toxicity (Fox et al. 1973).

Cysteine is a dispensable amino acid that can be synthesized in the body from Met (sulfur donor) and serine (carbon and nitrogen donors). It is dispensable, however, only when Met is present in the diet at levels sufficient to meet the entire total sulfur amino acid requirement. The intracellular concentration of cysteine ranges from 30 to 200 μM (Tateishi et al. 1977). GSH synthesis is regulated by availability of cysteine. Cysteine and cysteine precursors have been shown to protect against xenobiotics-induced toxicity by increasing GSH reserves (Williamson et al. 1982).

There are several endogenous sulfur containing compounds of toxicological importance that require cysteine for their synthesis. These include GSH and MT. MT, a low molecular weight, cysteine-rich protein, and GSH, a γ-Glu-CysH-Gly peptide, are widely distributed in nature. In many respects, both molecules appear to be closely related. Thus, MT and GSH present extra- and intracellularly in many tissues and are capable of binding heavy metals such as Cd. One-third of the amino acids present in MT and GSH are cysteine. Moreover, increased cellular MT or GSH levels may protect cells against oxidative damage, toxic

compounds and radiation. The increased flow of sulfur containing amino acids into the liver could be used for liver MT synthesis. Hepatic GSH concentration is highly dependent upon dietary levels of sulfur containing amino acids (Tateishi et al. 1981). Dietary restriction of sulfur containing amino acids results in depletion of hepatic GSH. Supplementation with Met or Cys had significantly elevated hepatic GSH (Goto et al. 1993). Hepatic GSH levels were decreased approximately 75% in rats fed diet containing 0.3% Met compared to the rats without Met in diet (Sendelbach et al. 1990).

When high levels of Met are fed to rats, pathological lesions develop in the liver, spleen, pancreas, small intestine and kidneys (Daniel and Waisman 1969). Diet containing 1% Met had only a slight growth depressing effect. It has been previously reported that there was an increased deposition of Fe in the spleen with high dietary Met. This is due to an increase in red blood cell turnover in rats fed on high levels of Met (Mengel and Klavins 1967). Consumption of excessive levels of Met results in depressed growth and tissue damage. The rats consuming diets containing 2% of Cys grow poorly and have tubular damage in the kidney and periportal necrosis in the liver (Benevenga et al. 1976).

We have employed the techniques of response-surface modelling (Cornell 1984) for the first time to determine the influence of dietary Met and Cys on Cd-induced biochemical changes in rats. Such models are often useful in providing a framework for suggesting possible directions for further research. The feasibility of the experiments can be enhanced through the use of central composite design, which requires fewer treatment combinations than conventional factorial design (Cornell 1984).

In the analytical approach employed, interactions were determined to occur between Met and Cys in the diet, depending on the statistical significance of the interaction term included

in the model equation. The agents are inferred not to interact (i.e., zero interaction) if the crossproduct is not significantly different from zero. Furthermore, the algebraic sign of the interaction term indicates whether the combined effect of Met and Cys is greater or less than the zero-interaction effect.

Models for the protective effects of Met and Cys against the Cd-induced biochemical changes have been determined and illustrated using regression coefficients and response surface plottings. A central composite rotatable design was used for the experimental settings for the nine groups of rats studied. With this experimental design, we have quantitatively estimated the effects of diet containing Met and Cys on Cd induced biochemical changes as well as the interactions between these two variables in rats treated with $CdCl_2$ (100ppm) in drinking water daily for 10 weeks .

In the present study, it has been shown that MT quantity of the liver was increased as the dietary Met increased at a higher dietary Cys intake. This is likely to be related to an increase in level of MT synthesis because cysteine-related pathways are involved in MT synthesis in the liver (Hidalgo et al. 1990).

The influences of diet containing Met and Cys either alone and in combination significantly increased the brain GSH content. The significant interactive effect between dietary Met and Cys on brain GSH content observed in this study may suggest that the sulfur containing amino acids increase GSH synthesis in brain. Because dietary levels of both Met and Cys are important in regulating GSH levels in the liver, it is possible that hepatic GSH transportation into brain will be increased as the dietary Met and Cys increased. In the testis only Cys alone increased the GSH contents, but in higher levels it seems to decrease testicular GSH. Results of this study along with our findings that the dietary Met and Cys produced a

significant increase in the brain and testis GSH levels signify the importance of sulfur containing amino acids in the *in vivo* biosynthesis of GSH. Cystine is easily converted to cysteine and increases the GSH formation. The findings of an increase in brain and testicular tissue levels of GSH suggest that dietary Met and Cys intake may offer protection against Cd-induced toxicity in these organs.

Met and Cys in the diet tended to increase Cd-induced lipid peroxidation as evident from a significant increase in brain MDA levels. A significant interaction between Met and Cys in the brain was observed at higher levels of their intake. Among the various effects induced by Cd in biological systems, the oxidative destruction of membrane polyunsaturated fatty acids, a phenomenon termed lipid peroxidation, has been observed in numerous tissues either *in vitro* and *in vivo* (Muller 1986; Vincent et al. 1989; Shukla et al. 1987; Hussain et al. 1987). In *in vitro* systems lipid peroxidation is an early intracellular event after Cd exposure although it does not seem to be generally critical to cell viability (Muller and Ohnesorge 1982). Any attempt to decrease lipid peroxidation may offer protection against Cd toxicity. Cysteine, Cys and Met are known to protect body tissue against oxidative damage (Arkrah et al. 1994). Feeding the rats on diets with Met reduced the susceptibility for lipid peroxidation by restoration of the level of free radical scavengers (Selvam and Kurien 1992). In our previous study, Cd injection significantly increased lipid peroxidation in testicular tissues of mice. The results of our present study failed to demonstrate that dietary Met and Cys significantly decreased MDA level, an index of lipid peroxidation. Cys in the diet had a significant quadratic effect on liver and testicular tissues MDA levels. However, diets containing both Met and Cys had significant interactive effects on brain lipid peroxidation in rats treated with 100ppm $CdCl_2$ in drinking water. Extensive literature search

has revealed that studies concerning the effects of dietary Met and Cys on Cd-induced lipid peroxidation are not existent. Modification of diets with various levels of Met and Cys may not be a reasonable approach to reduce or prevent Cd-induced lipid peroxidation of vital organs in the body. Further studies will be necessary in optimizing the levels of Met and Cys either alone or in combination in the diet which would minimize Cd-induced lipid peroxidation in any vital organs.

Cd interacts with functional sulfur groups of structural and functional cell membranes and inhibits the membrane bound ATPase enzymes, necessary for the active transport of Na^+, Ca^{2+} and Mg^{2+} ions. In this study, a combination of Met and Cys in the diet for 10 weeks didn't produce significant changes in intestinal mucosal Na^+,K^+-ATPase and total ATPase. However, dietary Met at higher levels significantly increased in Mg^{2+}-ATPase activity in the intestinal mucosa. The significance of an increase in Mg^{2+}-ATPase activity in the intestinal mucosa of the rats fed on high dietary Met is unknown at this time.

Aspartate(AST) and alanine(ALT) aminotransferases are intracellular amino-transferring enzymes present in large quantities in liver cells. Following injury or death of liver cells, they are released into the circulation. In general, the serum transaminases are sensitive tests of liver damage, and an increase of the serum transaminase activity reflects the severity of hepatic necrosis. AST is more specific for liver cell damage and AST sometimes reflects cardiac or skeletal muscle necrosis.

An increase in AST activity is seen in rabbits given 0.25mg Cd/kg 5 days a week for 11 weeks. Cd can elevate AST and alkaline phosphatase levels and this is related to Cd-induced changes in the rough endoplasmic reticulum in the liver (Samarawickrama 1979). Cd also accumulates in the myocardium and in the walls of greater vessels and may lead to damage

of heart and blood vessels (Samarawickrama 1979). Oral administration of the Cd, either in drinking water or in food, causes cardiac hypertrophy. In the present study, Cys has a significant quadratic effect on Cd-induced AST and Met had a significant quadratic effect on Cd-induced ALT. It seems that Cys and Met in high levels in the diet have an additive effect on Cd-induced liver damage as reflected by a significant increase in serum AST.

Response surface regression analysis revealed that dietary levels of Met and Cys did not have any significant influence on most of the clinical chemistry profiles in Cd treated rats. Perhaps, the explanations for this observation are: 1) there is a wide variation from rat to rat in the clinical chemistry data and 2) the second-degree rotatable model used in this study may not have included all appropriate functions of independent variables (Box and Draper 1987; Torreggiari et al. 1995).

In summary, with the limited number of experimental animals and response surface analysis technique, the results of our study suggest that the manipulation of dietary Cys/Met could produce a significant increase in liver MT and brain GSH in rats treated with $CdCl_2$ (100ppm) in drinking water daily for 10 weeks. These effects appear to offer protection against Cd-induced toxicoses. A significant increase in lipid peroxidation and serum AST and ALT suggest that modification of diets with various levels of Met and Cys alone and in combination may not be a good approach in minimizing these Cd-induced biochamical changes.

REFERENCES

Akera T and Brody TM (1969) The effects of ouabain on sodium- and potassium-activated adenosine triphosphatase from the hearts of several mammalian species. J Pharma Exp Ther 170:17-26

Ankrah NA, Rikimaru T, Ekuban FA and Addae MM (1994) Decreased cysteine and glutathione levels: possible determinants of liver toxicity risk in Ghanaian subjects. J Int Med Res 22:171-176

Benevenga NJ, Yeh MH and Laligh JJ (1976) Growth depression and tissue reaction to the consumption of excess dietary methionine and s-methyl-L-cysteine. J Nutr 106:1714-1720

Bousquet WF (1979) Cadmium toxicity. Vol. 15: Cardiovascular and renal effects (Mennear JH ed.). Marcel Dekker, Inc., New York. pp. 133-157

Box GEP and Draper NR (1987) Empirical model building and response surfaces. John Wiley and Sons, Inc., New York. pp. 73-94

Broekhuysen J, Clinet M and Delisee C (1972) Action of amiodarone on guinea pig heart sodium and potassium activated adenosine triphosphatase, comparison with ouabain. Biochem Pharmacol 21:2951-2960

Chen CL and Sangiah S (1992) Effects of BRB-1-28, a novel antiarrhythmic agent, and its derivatives on cardiac Na^+, K^+-ATPase, Mg^{2+}-ATPase activities and contractile force. Res Comm Chem Path Pharm 78:3-16

Cornell JA (1984) How to apply response surface methodology (Cornell JA and Shapiro SS eds). American Society for Quality Control, Milwaukee, Wisconsin. pp. 32-34

Covanni M (1983) Response surface metholodology and product optimization. Food Tech 37:41-45

Daniel RG and Waisman HA (1969) Adaptation of the weanling rat to diets containing excess methionine. J Nutr 99:299-306

Dudley RE and Klaassen CD (1984) Changes in hepatic glutathione concentration modify cadmium-induced hepatotoxicity. Toxicol Appl Pharmacol 72:530-538

Elinder CG and Nordberg M (1985) Cadmium and health: A toxicological and epidemogical appraisal: Metallothionein. (Frberg L, Elinder CG, Kjellstrom T and Nordberg F Eds). Vol. I. C.R.C. Press, Florida. pp.65-75

Elinder CG (1986) Cadmium and health: A toxicological and epidemic appraisal: other toxic effects (Friberg L, Elinder CG, Kjellstrom T and Nordberg G Eds). Vol. II, C.R.C. Press, Florida pp.159-195

Fox MRS, Jacobs RM, Fry BE and Harland BF (1973) Effect of protein source on response to cadmium. Fed Proc 32:924-927

Garcia RAG and Stipanuk MH (1992) The splanchnic organs, liver and kidney have unique roles in the metabolism of sulfur amino acids and their metabolites in rats. J Nutr 122:1693-1701

Georce JR, Harbers LH and Reeves RD (1980) Digestive responses of rats to fiber type, level and particle size. Nutr Rep Int 21:312-322

Goto H, Yoshida M and Nakazono N (1993) Effect of dietary protein and sulfur-containing amino acids on urinary metabolites in rats injected with chlorobenzene. J Toxicol Sci 18:103-109

Gunn SA, Gould TC and Anderson WAD (1966) Protective effect of thiol compounds against cadmium-induced vascular damage to testis. Proc Soc Exp Biol Med 122:1036-1039

Gunn SA, Gould TC and Anderson WAD (1968) Mechanisms of zinc, cysteine and selenium protection against cadmium-induced injury to mouse testis. Reprod Fert 15:65-70

Hidalgo J, Garvey JS and Armario A (1990) On the matallothionein, glutathione and cysteine relationship in rat liver. J Pharm Ther 255:554-564

Hussain T, Shukla S and Chandra SV (1987) Effect of cadmium on superoxide dismutase and lipid peroxidation in liver and kidney of growing rats. Pharmacol Toxicol 60:355-363

Kagi JHR and Vallee BL (1960) Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. I. J Biol Chem 235:3460-3465

Klassen CD, Dudley RE, Goering PL, Hazelton GA and Hjelle JJ (1985) Role of sulfhydryls in the hepatotoxicity of organic and metallic compounds. Fundam Appl Toxicol 5:806-815

Kosower NS and Kosower EM (1978) The glutathione status of cells. Int Rev Cytol 54:109-155

Mahaffey KR, Corneliussen PE, Jelinek CF and Fiorino JA (1975) Heavy metal exposure from foods. Environ Health Perspect 12:63-68

Mengel CE and Klavins JV (1967) Development of hemolytic anemia in rats fed methionine. J Nutr 92:104-110

Muller L and Ohnesorge FK (1982) Different responses of liver parenchymal cells from starved and fed rats to cadmium. Toxicol 25:141-146

Muller L (1986) Consequences of cadmium toxicity in rat hepatocytes: mitochodral dysfunction and lipid peroxidation. Toxicol 40:285-291

Ohkawa H, Ohishi N and Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351-358

Samarawickrama GP (1979) The chemistry, biochemistry and biology of cadmium: Biological effects of cadmium in mammals (Webb M. Ed.) Elsevier/North-Holland Biomedical Press, New York pp. 360-373

Schutz HG (1983) Multiple regression approach to optimization. Food Tech 37:46-62

Sendelbach LE, White CA, Howell S, Gregus Z and Klaassen CD (1990) Effect of sulfhydryl-deficient diets on hepatic metallothionein, glutathione, and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) levels in rats. Toxicol Appl Pharmacol 102:259-267

Selvam R and Kurien TB (1992) Restoration of antioxidants in liver by methionine feeding in experimental rat urolithiasis. Indian J Biochem Biophys 29:364-370

Shen Y and Sangiah S (1994) Influence of cadmium on ketamine-induced anesthesia and brain microsomal Na^+, K^+-ATPase in mice. Bull Environ Contamin Toxicol 53:591-597

Shukla GS, Hussain T and Chandra SV (1987) Possible role of regional superoxide dismutase activity and lipid peroxides levels in cadmium neurotoxicity: in vivo and in vitro studies in growing rats. Life Sci 41:2215-2219

Sies H and Wendel A (1978) Functions of glutathione in liver and kidney. Springer-Verlay, Berlin.

Singhal RK, Anderson ME and Meister A (1987) Glutathione, a first line of defense against cadmium toxicity. FASEB J 1:220-223

Tallarida RJ and Murray RB (1986) Manual of pharmacologic calculations with computer programs. Tallarida RJ, Springer-Verlag, New York, pp. 131-134

Taniguchi M and Cherian MG (1990) Ontogenic changes in hepatic glutathione and metallothionein in rats and the effect of a low-surphur-containing diet. Br J Nutr 63:97-103

Tateishi N, Higashi T, Naruse A, Nakashima K, Shiozaki H and Sakamoto Y (1977) Rat liver glutathione: possible role as a reservoir of cysteine. J Nutr 107:51-60

Tateishi N, Higashi T, Nause A, Hikata K and Sakamote Y (1981) Relative contributions of sulfur atoms of dietary cysteine and methionine to rat liver glutathione and proteins. J Biochem 90:1603-1610

Teare JP, Punchard NA and Powell JJ (1993) Automated spectrophotometric method for

determining oxidized and reduced glutathione in liver. Clin Chem 39:686-689

Torreggiani D, Toledo RT and Bertold G (1995) Optimization of vapor induced puffing in apple dehydration. J Food Sci 60:181-185

Toyomizu M, Akiba Y, Matsumoto T and Horiguchi M (1985) Response surface analysis of body protein and energy gains in growing chicks fed diets over the entire range of compositions of protein, fat and carbohydrate. J Nutr 115:61-69

Vincent R, Boudreau J, Nadeau D, Fournier M, Kryzystyniak K and Trottier B (1989) Lipid peroxidation in rat lungs following an acute inhalation exposure to cadmium chloride. J Aer Med 24:349-357

Williamson JM, Boettcher B and Meister A (1982) Interacellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proc Natl Acad Sci USA 79:6246-6249

TABLE 3

MT levels (Mean ±SE) of liver and kidney
of rats fed on the experimental diets containing variable levels of
Met and Cys with $CdCl_2$ (100ppm) in drinking water for 10 weeks

| | % of diet | | MT(µg/g) | |
diet no.	Met	Cys	liver	kidney
01	0.22	0.12	47.48± 6.31	10.71±2.41
02	1.28	0.12	46.93± 6.31	14.95±2.60
03	0.22	0.68	62.72± 5.73	12.94±2.71
04	1.28	0.68	85.01± 0.79	12.72±3.54
05	0.00	0.40	50.53±14.52	13.29±2.03
06	1.50	0.40	53.78±13.56	11.36±2.11
07	0.75	0.00	63.26±10.41	11.50±1.54
08	0.75	0.80	73.30±14.44	15.60±3.96
09	0.75	0.40	58.62± 4.51	14.63±1.72

TABLE 4

GSH contents (Mean ±SE) of brain, heart, liver and testicular
tissues of rats fed on the experimental diets containing variable levels
of Met and Cys with $CdCl_2$ (100ppm) in drinking water for 10 weeks

diet no.	% of diet		GSH(µg/100mg)			
	Met	Cys	brain	heart	liver	testis
01	0.22	0.12	87.54±20.08	210.68± 6.10	582.32± 39.95	234.36± 4.11
02	1.28	0.12	106.83±22.24	216.17± 3.68	634.76± 44.08	188.55±10.61
03	0.22	0.68	65.82±14.23	218.19±12.26	545.56± 21.18	184.42±27.53
04	1.28	0.68	131.41± 4.63	204.27± 9.19	366.10± 31.20	179.10± 9.59
05	0.00	0.40	89.93± 5.01	214.89± 8.14	521.73± 60.05	219.34±20.98
06	1.50	0.40	109.99±10.59	220.57± 1.55	599.05± 56.11	237.71± 5.33
07	0.75	0.00	84.99±14.30	205.77±15.92	494.80± 50.48	324.48±26.97
08	0.75	0.80	112.33±13.15	223.02± 8.47	696.45± 46.04	300.94±19.13
09	0.75	0.40	98.72± 3.43	210.22± 7.09	620.67± 74.28	324.67±20.30

TABLE 5

MDA levels(Mean ±SE) of brain, liver and testicular tissues
of rats fed on the experimental diets containing variable levels
of Met and Cys with CdCl (100ppm) in drinking water for 10 weeks

	% of diet		MDA(nmol/g)		
Diet no.	Met	Cys	brain	liver	testis
01	0.22	0.12	143.97± 3.43	188.77±21.60	119.03±2.88
02	1.28	0.12	127.62±10.32	195.45± 1.43	121.83±7.36
03	0.22	0.68	121.90± 5.23	217.74±13.58	119.96±5.72
04	1.28	0.68	149.05± 0.75	211.97± 0.49	119.49±5.87
05	0.00	0.40	145.65± 1.75	185.50± 6.83	120.89±0.66
06	1.50	0.40	137.12± 4.10	212.32± 5.18	126.97±3.03
07	0.75	0.00	138.05± 1.15	226.32± 8.75	122.76±4.12
08	0.75	0.80	140.92± 3.06	255.75±10.48	113.89±6.30
09	0.75	0.40	137.45± 2.26	228.22± 11.80	112.47±6.08

TABLE 6

ATPase activity (Mean ±SE) of intestinal mucosa
of rats fed on the experimental diets containing variable
levels of Met and Cys with CdCl$_2$ (100ppm) in drinking water for 10 weeks

	% of diet		ATPase(nM pi/mg protein/min)		
Diet no.	Met	Cys	total ATPase	Na+,K+-ATPase	Mg2+-ATPase
01	0.22	0.12	421.07±43.88	144.26±20.84	276.81±30.93
02	1.28	0.12	351.85± 9.85	134.25± 5.34	217.59±10.51
03	0.22	0.68	311.82±32.52	110.57± 5.61	201.25±31.36
04	1.28	0.68	292.21±37.86	125.12± 0.64	170.77±32.67
05	0.00	0.40	301.16±44.42	159.71±32.36	139.44±14.91
06	1.50	0.40	294.09±23.39	151.57± 8.93	142.52±18.04
07	0.75	0.00	300.55±78.80	149.10±19.97	214.79±37.72
08	0.75	0.80	303.44± 9.03	136.50±16.74	166.94±21.20
09	0.75	0.40	308.13±10.04	138.62± 6.99	169.51± 8.93

FIGURE LEGENDS

Fig. 1: Three-dimensional plot of liver MT(nmol/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 55.92 - 43.86x1 +9.87x2 + 59.28x1^2 -11.80x2^2 + 33.30x1x2.

Fig. 2: Three-dimensional plot of brain GSH (μg/100mg tissue) as function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 94.15 - 35.08x1 - 5.32x2 - 5.28x1^2 + 0.79x2^2 + 76.64x1x2.

Fig. 3: Three-dimensional plot of testis GSH (μg/100mg tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 192.04 + 121.74x1 + 307.16x2 - 289.29x1^2 - 233.11x2^2 + 103.96x1x2.

Fig. 4: Three-dimensional plot of brain lipid peroxidation (nmol MDA/g tissue) as a function of percent of Met(x1) with percent of Cys(x2)in the diet. y = 160.36 - 52.00x1 - 34.30x2 - 2.79x1^2 + 2.87x2^2 + 74.78x1x2.

Fig. 5: Three-dimensional plot of liver lipid peroxidation (nmol MDA/g tissue) as a function of percent of Met(x1) with percent of Cys(x2)in the diet. y = 168.87 + 23.06x1 + 113.54x2 + 33.84x1^2 - 65.51x2^2 + 12.21x1x2.

Fig. 6: Three-dimensional plot of testis lipid peroxidation (nmol MDA/g tissue) as a function of percent of Met(x1) with percent of Cys(x2)in the diet. y = 127.37 - 27.75x1 - 23.82x2 + 32.93x1^2 + 19.31x2^2 - 6.91x1x2.

Fig.7: Three-dimensional plot of intestinal mucosa Mg$^+$-activated ATPase (nm pi/mg protein/min) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 283.91 - 374.64x1 - 46.67x2 + 300.66x1^2 - 0.46x2^2 + 76.13x1x2.

Fig. 8: Three-dimensional plot of ALT (IU/L) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 67.15 - 145.00x1 + 11.17x2 + 297.66x1^2 + 63.18x2^2 - 132.89x1x2.

Fig. 9: Three-dimensional plot of AST (IU/L) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 300.79 - 416.76x1 - 253.73x2 + 664.35x1^2 + 302.55x2^2 - 95.00x1x2.

Fig. 10: Three-dimensional plot of creatinine(mg/dl) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. y = 0.52 + 0.67x1 + 0.15x2 - 0.74x1^2 - 0.06x2^2 - 0.18x1x2.
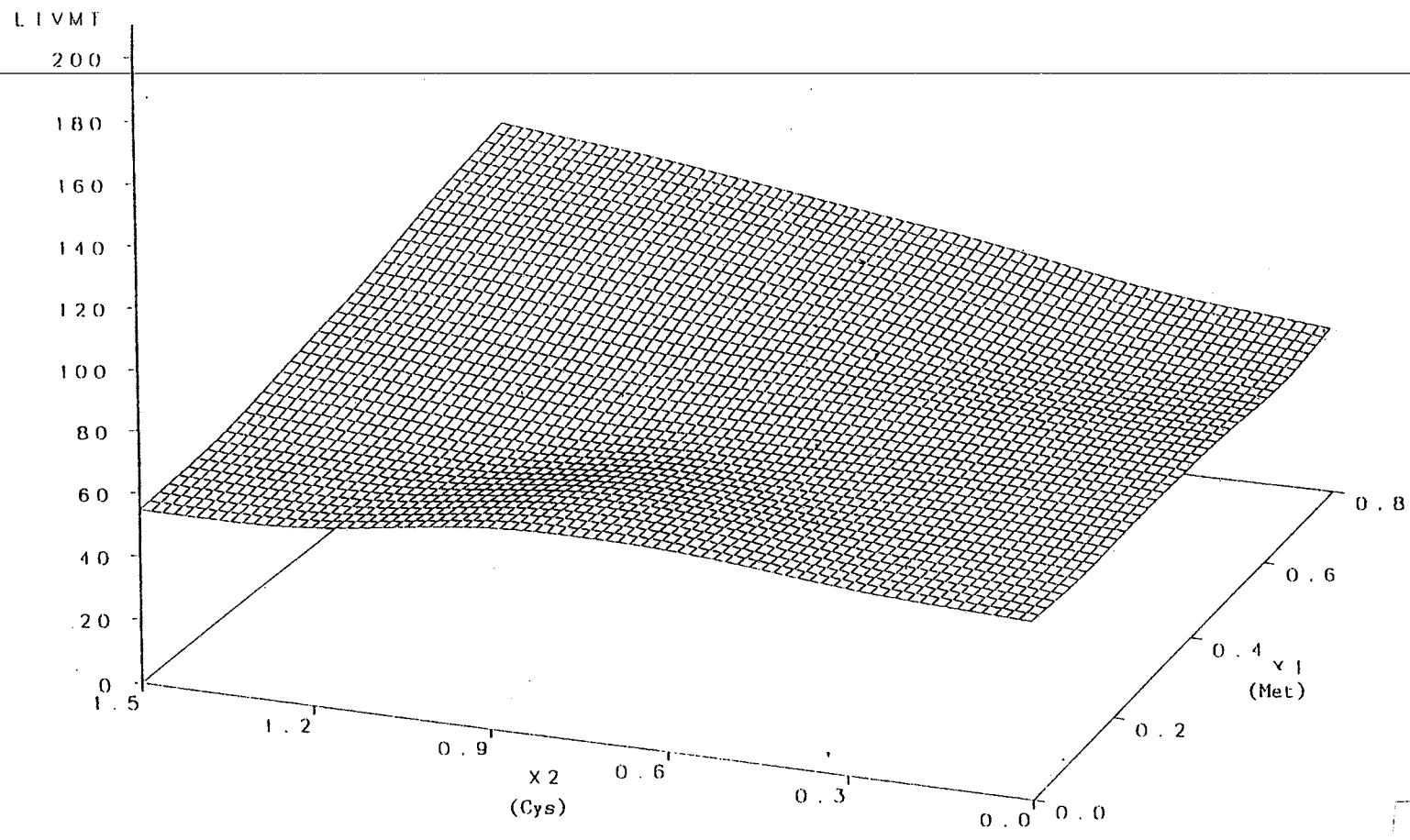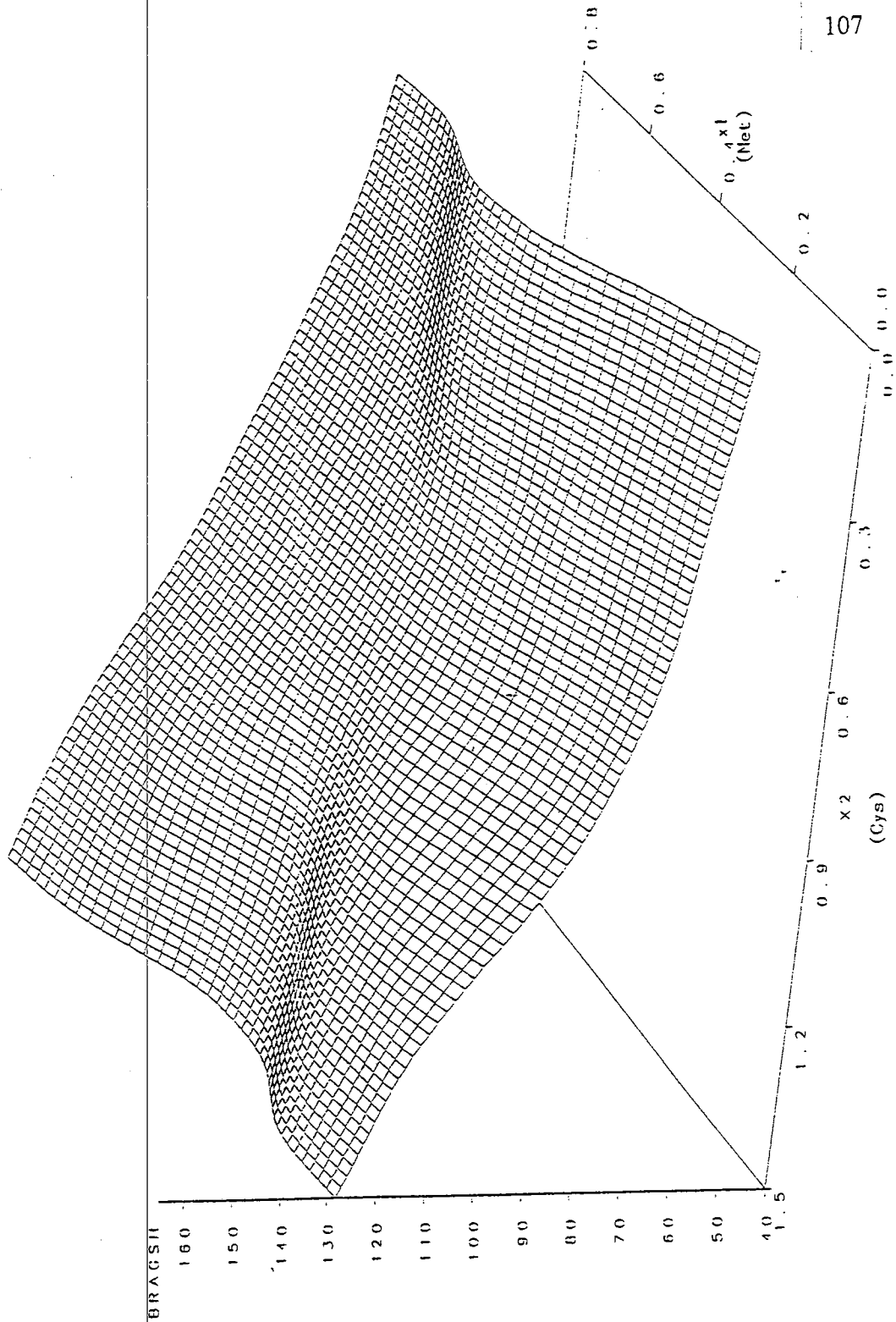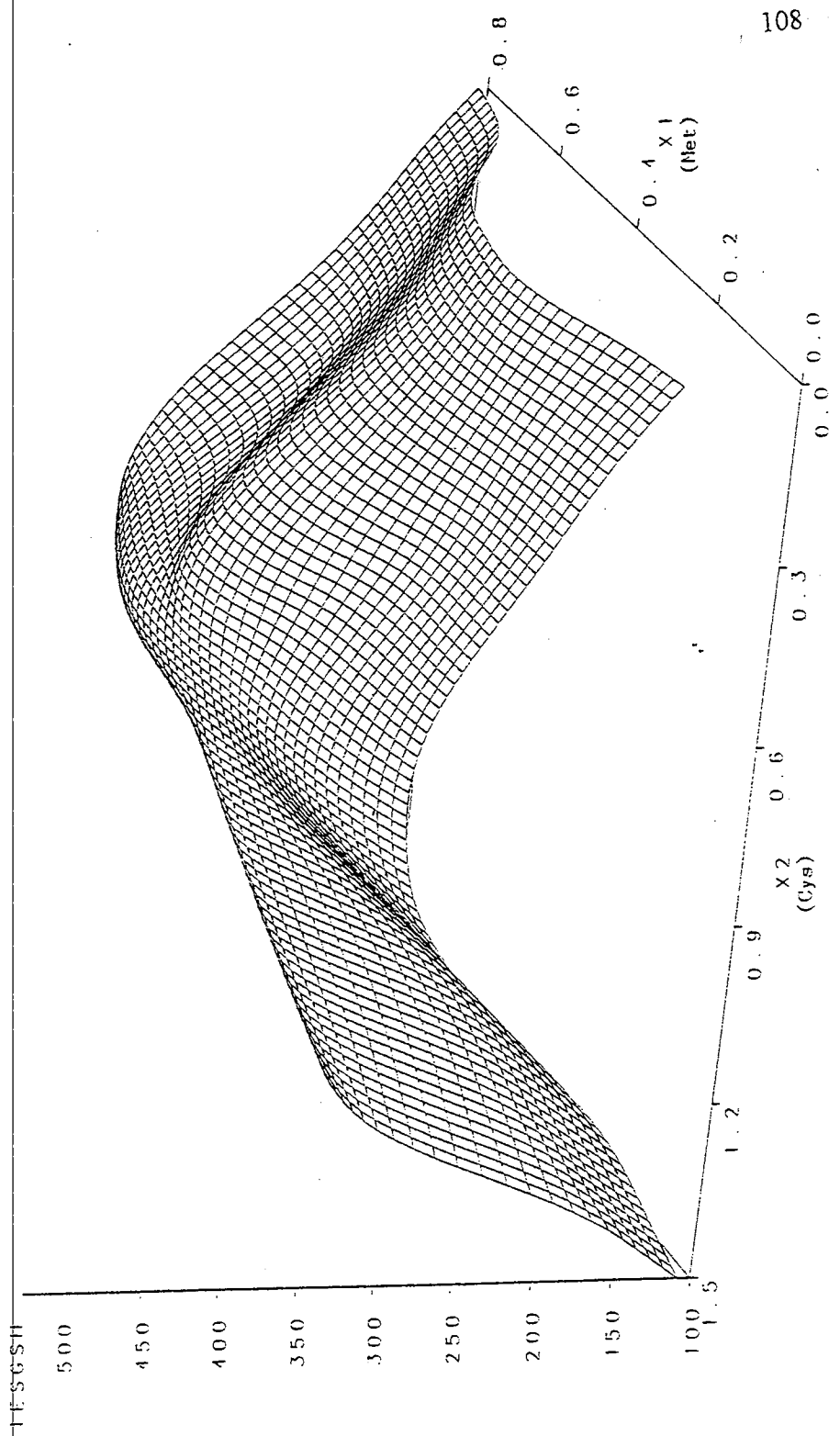
Fig. 1

Fig 2

Fig. 3
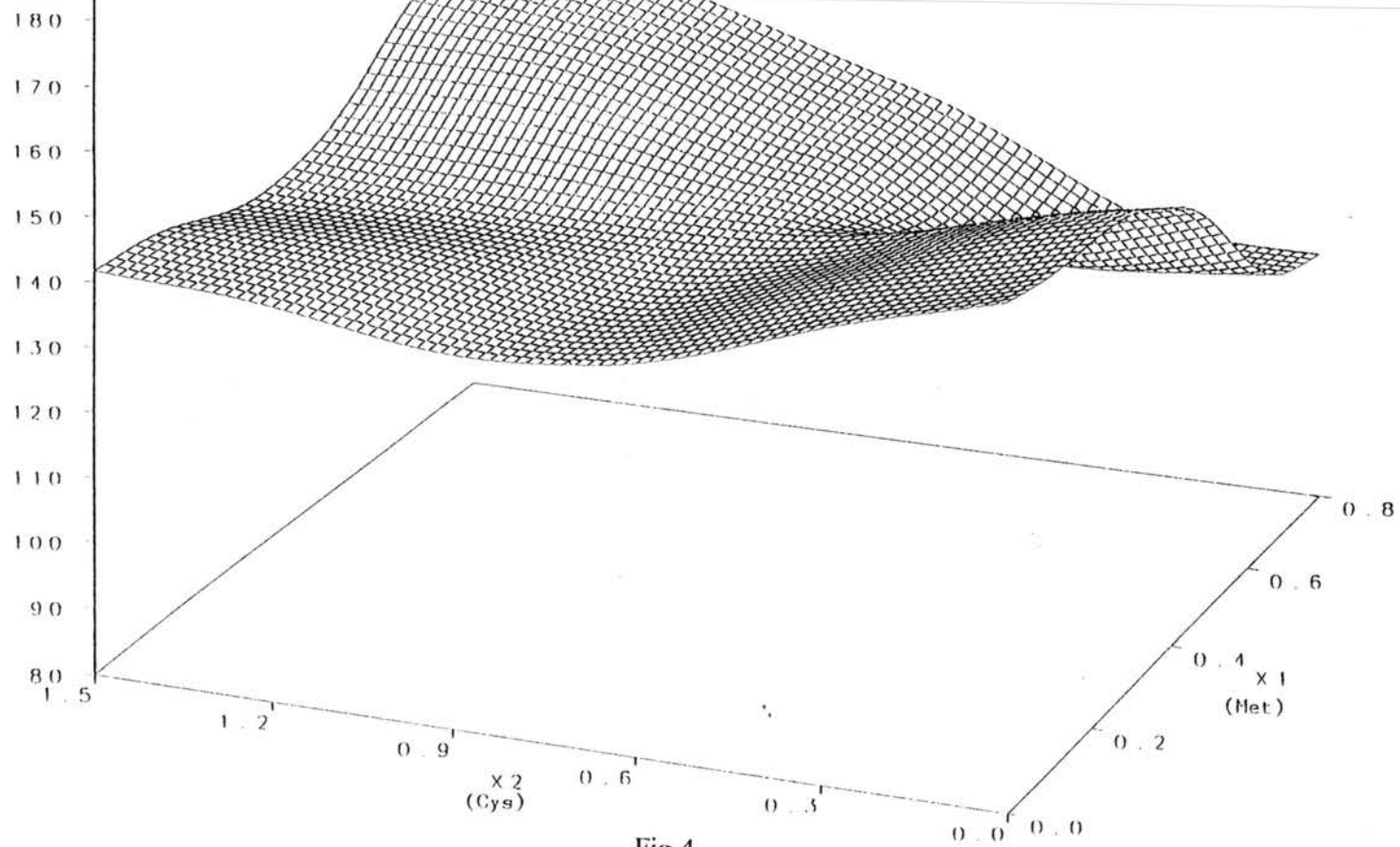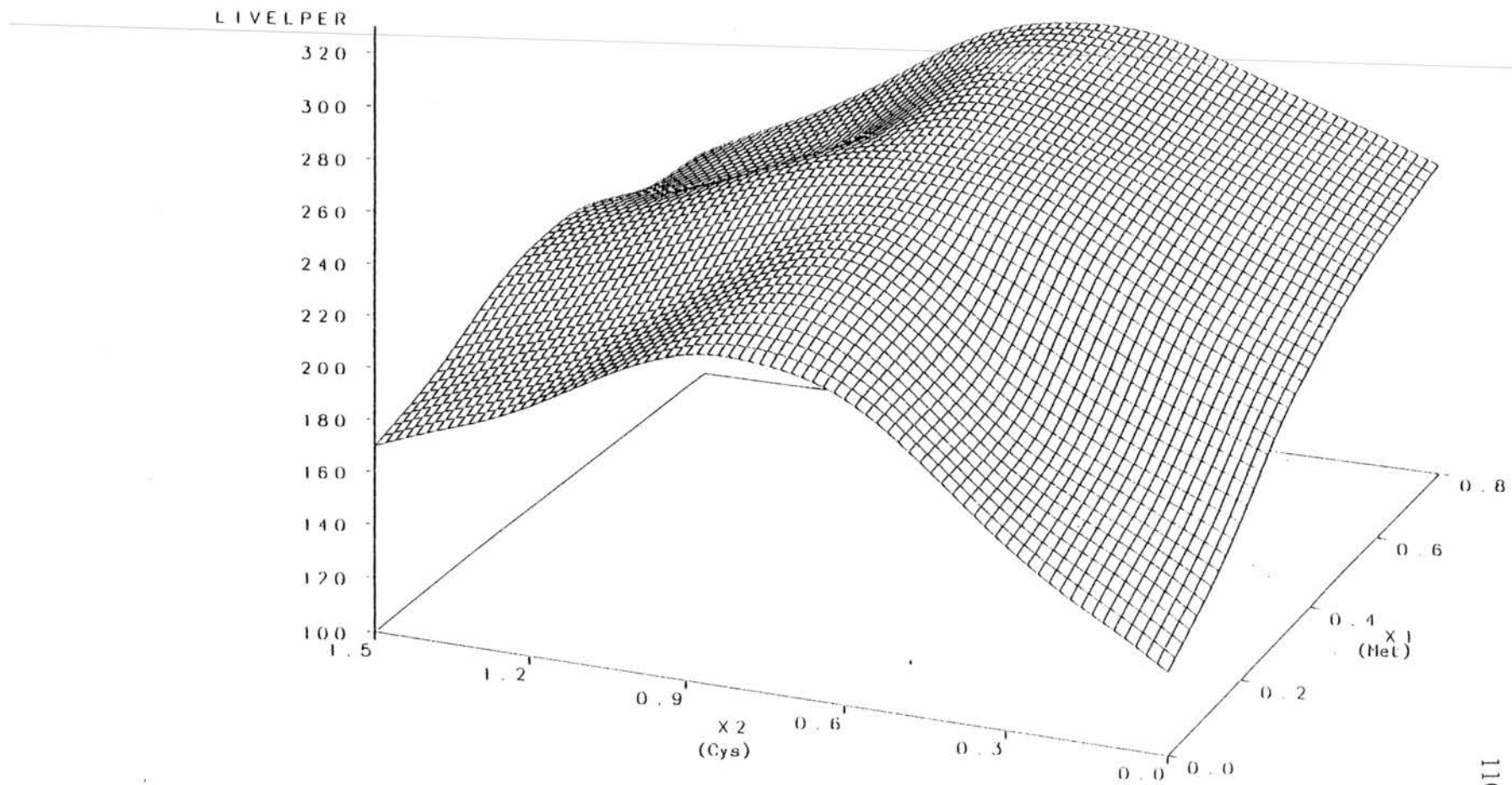
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Fig. 5

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Fig. 8

Fig.9

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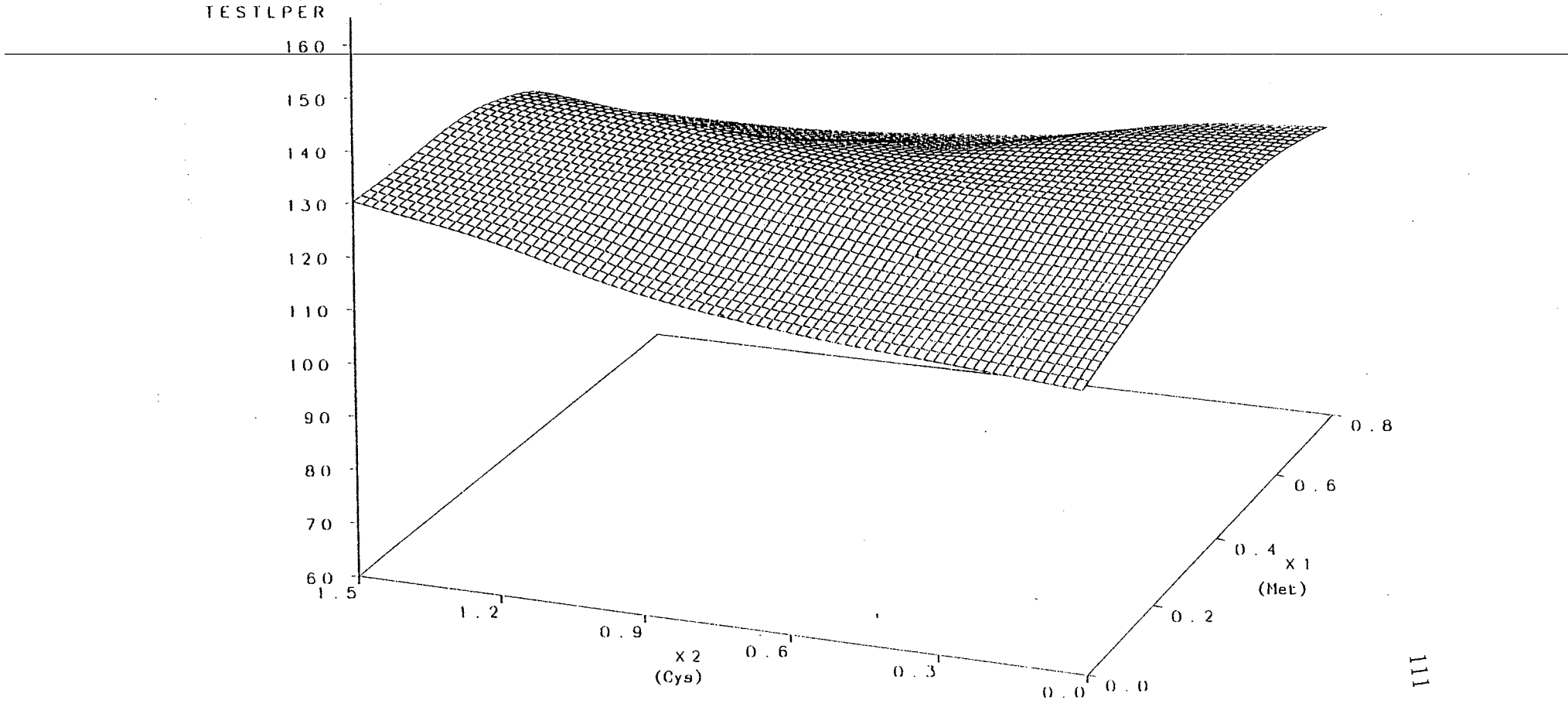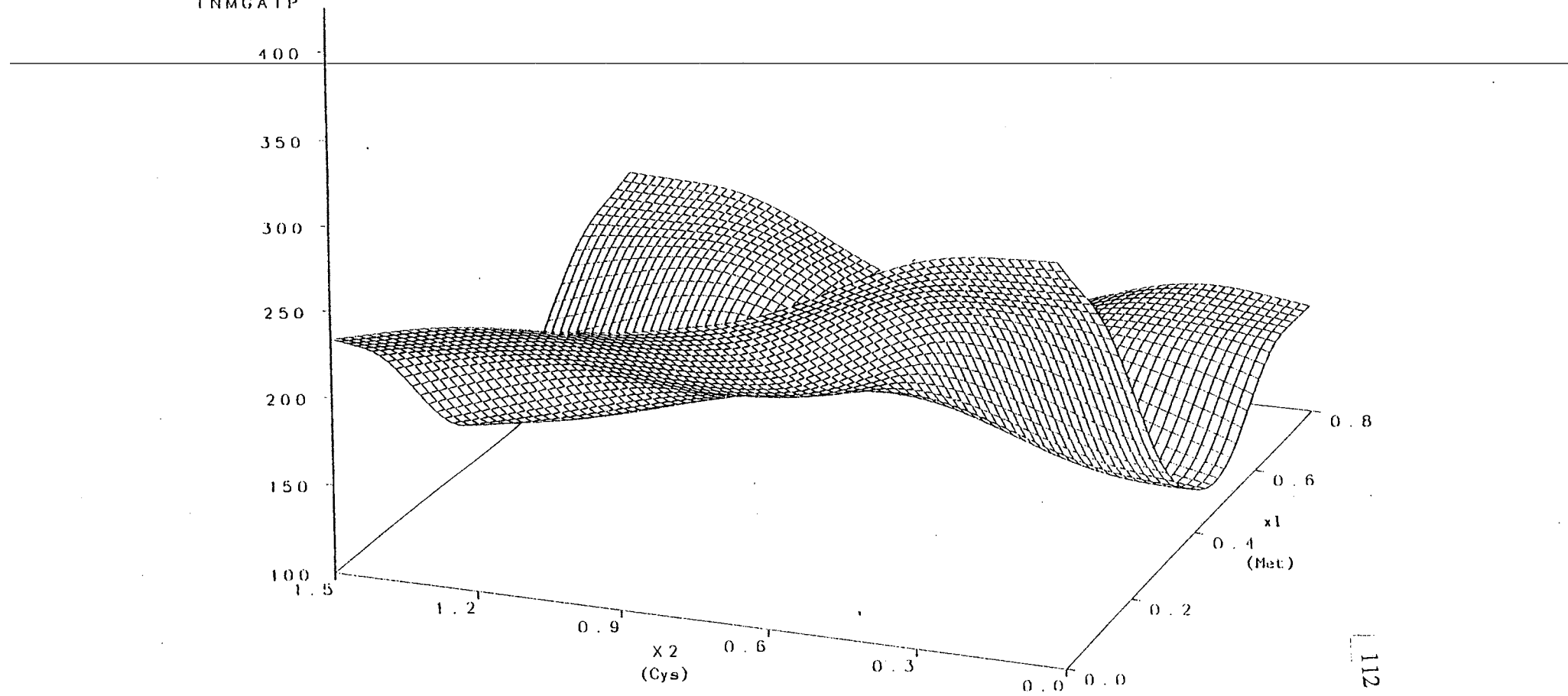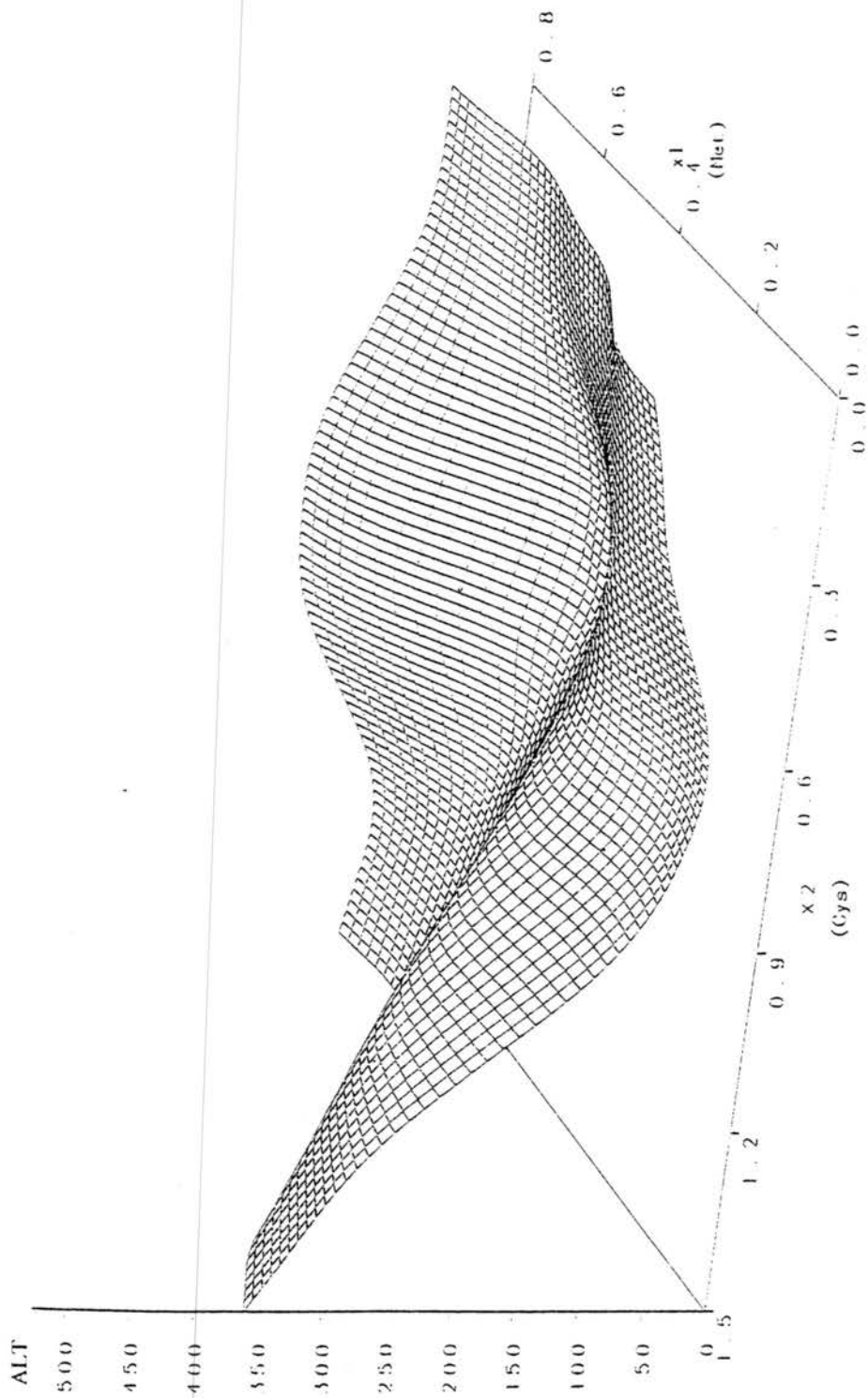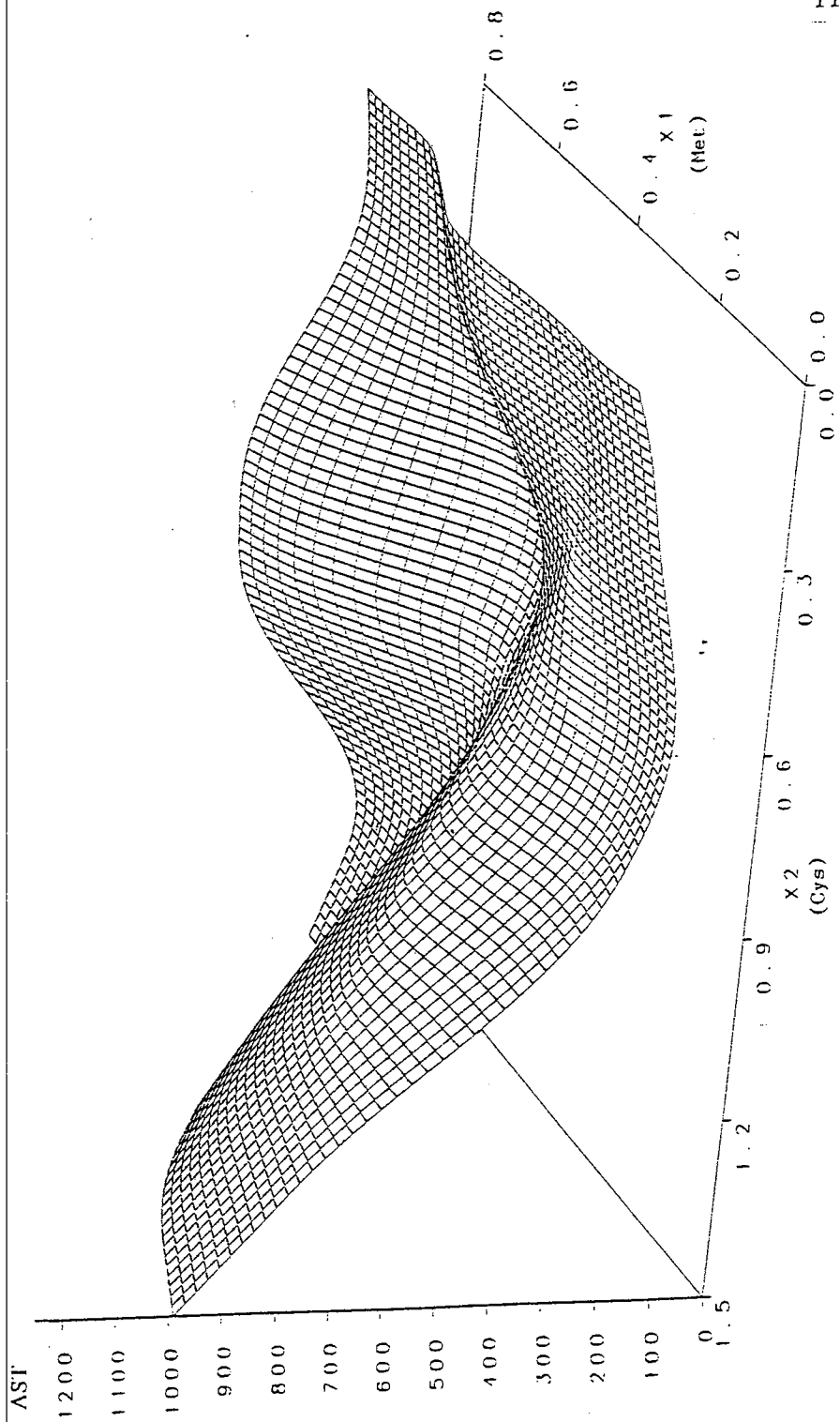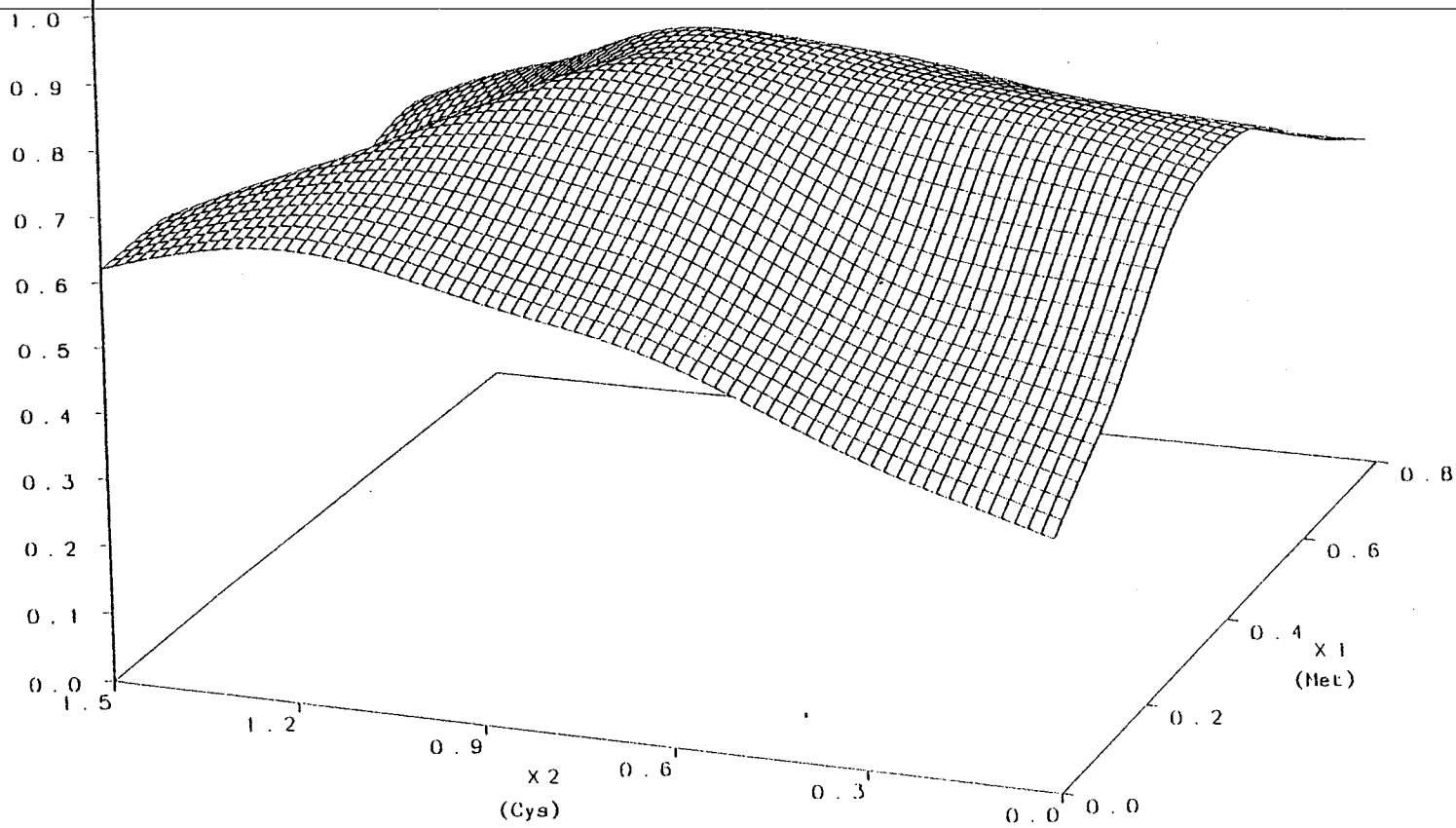
Fig. 10

CHAPTER V

RESPONSE SURFACE ANALYSIS OF EFFECTS OF DIETARY SULFUR CONTAINING AMINO ACIDS ON CADMIUM AND CADMIUM INDUCED CHANGES IN COPPER, IRON AND ZINC DISTRIBUTION

ABSTRACT

Response surface regression analysis was used to study the effects of dietary levels of methionine(Met) and cystine(Cys) on the distribution of cadmium(Cd), copper(Cu), iron(Fe) and zinc(Zn) in the liver, kidney and testis of rats. The regression coefficients and estimated parameters of these response surfaces and three dimensional plots were developed to help evaluate the effects of Met and Cys either alone or in combination on Cd and Cd-induced changes in Cu, Fe and Zn distribution. Rats were randomly assigned to one of nine experimental diets containing variable concentrations of Met (0.00 to 0.80g/100g diet) and Cys(0.00 to 1.5g/100g diet). The individual rats within each experimental group received $CdCl_2$(100ppm) in drinking water daily for 10 weeks. After 10 weeks, the liver, kidney and testis were collected for Cd, Cu, Fe and Zn analysis. Dietary Met and Cys had significant effects on Cd distribution in the liver, kidney and testis. They also had significant influences on testis Cu, liver Fe, kidney and testis Zn levels. These results are discussed in relation to the influence of dietary sulfur containing amino acids on changes in the levels of metallothionein(MT) in the liver and its link to Cd-induced toxicity in animals.

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INTRODUCTION

Cd is a major occupational and environmental pollutant. Humans are exposed to Cd through inhalation of ambient air, cigarette smoke and emission from combustion of fuels and plastic waste. Industrial workers are exposed to Cd in metal smelter, alloy, paint-pigment, battery, ceramic, textile-printing and welding industries (Friberg et al. 1974; Nriagu 1980). Cd is present in almost all types of food. Shellfish, wheat and rice accumulate very high amounts of Cd (Fielder and Dale 1983). Thus, besides environmental or occupational exposure, the main source of Cd exposure in the general population is diet. Exposure to Cd causes anemia, hypertension, hepatic, renal and cardiovascular disorders (Flick et al. 1971; Friberg et al. 1976).

The interactions of Cd with certain trace elements can produce symptoms characteristic of trace element deficiencies. As a result, the symptoms or the clinical signs of chronic Cd toxicity in certain animal species closely resemble those of Zn and/or Cu deficiency and can be prevented by administering higher doses of the salts of these trace elements (Petering et al. 1979; Mills and Delgarno 1972).

There are complex antagonistic interactions of Cd, Cu and Zn in animals. Both the concentration and distribution of Cd in tissues can be altered by Cu and Zn under experimental conditions. Marginal Zn deficiency increased the incidence of Cd-induced injection site sarcomas and enhanced the progression of testicular tumors after a single high s.c. dose of Cd (Waalkes et al. 1991). Exposure of rats to Cd depresses serum Zn at all levels of Zn nutrition (Samarawickrama 1979). Se and Zn can protect against hepatotoxic effects of Cd. Zn deficiency in animals affects the gonads, and it has been found that Cd

administration can displace significant amounts of testicular Zn. Cd inhibits the absorption of Cu and Fe and affects Cu metabolism. Fe also inhibits Cd absorption. Fe deficiency has been shown to increase gastrointestinal absorption of Cd in humans (Flanagan et al. 1978). In Japanese quail, Cd toxicity was intensified by single or combined deficiencies of Zn, Cu or Fe (Fox et al. 1979).

Sulfur containing amino acids such as cysteine, cystine and methionine play an important role in Cd toxicity. Cysteine has been shown to change the transportation and distribution of Cd and reverse testicular damage in mice (Gunn et al. 1966). Cystine occurs normally in our food intakes. Cysteine and cystine are in fact easily interconvertible in the body. Other sulfur containing amino acids also are related to cysteine metabolism. The sulfur of cysteine is derived from methionine.

The objective of this study is to determine whether or not diets with different levels of sulfur containing amino acids such as Cys and Met will change Cd distribution in target organs and to study the impact of Cd on Cu, Zn and Fe distribution in rat liver, kidney and testicular tissues.

MATERIALS AND METHODS

Chemicals and Regents

All the reagents used in this study were of analytical grade. $CdCl_2$ was obtained from Sigma Chemical Co. (St Louis, MO). Nitric acid was purchased from Merck (Rahway, NJ). Standard solutions of Cd, Zn, Cu and Fe were purchased from Inorganic Ventures, Inc. (Brick, NJ).

<u>Animals</u>

Thirty nine, 4 weeks old, mycoplasma and specific virus-antigen-free, male Sprague-Dawley (Sasco Labs., Omaha, NE) rats (120-140g) were used in this study. They were housed in individual stainless steel cages with wire mesh floors at constant temperature, humidity, and air circulation according to the Guide for Care and Use of Laboratory animals (NIH, 1985). They were kept on a 12 hr light-dark cycle. One week after arrival, the rats were randomly assigned to 9 different treatment groups.

<u>Diets</u>

Basal (Harlan Teklad, Madison, WI) diets with the following compositions (Table 1) were mixed with different levels of cystine and methionine in powder form. The diets were fed to the individual rats within the treatment groups.

Various levels of DL-Methionine(Met) and L-Cystine(Cys) were added to the basal diet according to the experimental design, presented in Tab. 2.

<u>Experimental Design</u>

The experimental design is a central composite rotatable design with two x-variables. Following one week adaptation with regular powder diet (containing 0.3% cystine added to the basal diet), the individual rats within the treatment groups were fed the experimental diets plus 100ppm $CdCl_2$ in drinking water daily for 10 weeks. Body weight and general conditions were recorded each week. Food and water consumptions were recorded every two days, throughout the 10 weeks period. All of the rats from each group were killed by decapitation following 24 hours starvation at the end of the 10 week period.

TABLE 1
MODIFICATION OF DIET AIN-93M
(Harlan Teklad)

ingredients	g/kg
CASEIN	140.0
SUCROSE	100.0
CORN STARCH	467.296---467.548
MALTODEXTRIN[1]	155.0
SOYBEAN OIL	40.0
CELLULOSE[2](fiber)	50.0
MINERAL MIX, AIN-93M-MX	35.0
VITAMIN MIX, AIN-93-VX	10.0
CHOLINE BITARTRATE	2.5
TBHQ[3] (antioxidant)	0.008

[1]Dextrinized corn starch (hydrolysate, 90-94% tetrasaccharides and higher).
[2]Solka-Floc[@], 200 FCC.
[3]tert-butylhydroquinone.

TABLE 2
Central composite rotatable design
and variable settings

rats	x1	x2	Met(x1) (g/100g diet)	Cys(x2) (g/100g diet)
3	-1	-1	0.12	0.22
3	-1	1	0.12	1.28
3	1	-1	0.68	0.22
3	1	1	0.68	1.28
3	0	$-\sqrt{2}$	0.40	0.00
3	0	$\sqrt{2}$	0.40	1.50
3	$-\sqrt{2}$	0	0.00	0.75
3	$\sqrt{2}$	0	0.80	0.75
15	0	0	0.40	0.75

Statistical Analysis of the Data

A two-factor central composite rotatable response surface design was used in these

experiments with the following equation:

$$y = b_0 + b_1 x1 + b_2 x2 + b_{11} x1^2 + b_{22} x2^2 + b_{12} x1 x2$$

The relationship between the measurements(trace elements), y, and the corresponding variable settings, x, is modeled. Here b_0 is the model intercept, x1 and x2 are the levels of methionine and cystine in rat diet, b_1 and b_{11} are parameters associated with the effects of methionine, b_2 and b_{22} are parameters associated with the effects of cystine, and b_{12} is the parameter associated with the interactions of methionine and cystine. The estimated parameters were determined using the PROC RSREG procedure of the Statistical Analysis System (SAS). In all analyses, P value < 0.05 was considered significant. Three dimensional response surface contour plots were produced for each variable using the three dimensional graphics procedure (G3D).

The optimal stepwise regression equation was selected for each variable, using the criteria that the last variable entered resulted in a significant improvement in the response surface regression analysis.

Determination of Cd, Cu, Fe and Zn in Liver, Kidney and Testicular Tissues

All of glassware were left in 10% HCl for 24 hours. They were washed and rinsed with deionized water. The individual rats of various treatment groups were sacrificed by decapitation with a guillotine. The liver, kidneys and testes were removed immediately. Tissue samples were collected from the individual rats organs and dried at 105°C in an oven and then wet ashed with HNO_3 and H_2O_2 at 105°C for one week in silica crucibles. Ash was dissolved in 0.5% HNO_3. Cd, Cu, Fe and Zn concentrations were determined after dilution by flame atomic absorption spectrophotometry (Perkin-Elmer Model 5100 PC spectrophotometer).

Cd concentration in testicular tissues were measured by graphite-furnace atomic absorption spectrophotometry (Perkin-Elmer Model 5100 PC spectrophotometer) (Jorhem 1993). Different concentrations of standard solutions of each metal were prepared to obtain standard calibration curves. The calibration curves were mostly linear over the range. The concentration of metal in sample was calculated by using the concentration in sample solution, mean concentration in blank solutions, volume of sample solution and dry weight of tissue sample.

RESULTS

Effects on Cd distribution

Cd distribution (mean±SE) in liver, kidney and testis of the rats fed on the experimental diets is presented in Tab. 3. Values for liver Cd concentration ranged from 34.0 to 140.5 μg/g tissue. The influence of dietary Met and Cys on Cd content in the liver has been demonstrated with a response surface plot (Fig. 1). From the response surface regression equation it was observed that Met(x1) had a significant effect ($P<0.05$) on liver Cd content. Using stepwise regression, the optimal regression equation was: y(liver Cd)=87.96-66.60Met(x1)+10.95Cys(x2)^2 ($P<0.05$).

Values for kidney Cd ranged from 59.0 to 133.0 μg/g tissue. From the response surface regression equation it was observed that both Met(x1) and Cys(x2) had significant linear effects($P<0.05$) on kidney Cd concentration (Fig. 2). When the data were analyzed by using stepwise regression, the optimal regression equation was: y(kidney Cd)=100.03-46.05Met(x1)+8.66Cys(x2)^2 ($P<0.05$).

Values for testicular tissues ranged from 0.51 to 2.95 µg/g tissue. From the response surface regression equation, it was observed that both Met(x1) and Cys(x2) had significant effects (P<0.05) on testicular Cd concentration (Fig.3). Using stepwise regression, the most predictive model using the criteria that the last variables entered resulted in a significant improvement for testis Cd was: $y(testis\ Cd)=1.48+0.72Cys(x2)^2-1.65Met(x1)Cys(x2)$ (P<0.05).

Effects on Cu distribution

Mean Cu concentrations in liver, kidney and testis for the rats fed on the experimental diets are presented in Tab. 4. Values for liver Cu ranged from 15.00 to 25.70 µg/g tissue and for kidney ranged from 31.00 to 83.05 µg/g tissue The response surface analysis has revealed that dietary Met and Cys did not have significant effects on Cd-induced changes in liver and kidney Cu. Values for testis Cu ranged from 7.00 to 13.6 µg/g tissue. The data indicated that dietary Cys(x2) had a significant quadratic effect (P<0.05) on testicular Cu (Fig. 4). Using stepwise regression, the most predictive model using the criteria that the last variables entered resulted in a significant improvement for Cu distribution in the testis was: $y(testis\ Cu)=13.28-6.56Cys(x2)+4.63Cys(x2)^2$ (P<0.05).

Effects on Fe distribution

Tab. 4 shows mean liver, kidney and testicular tissue Fe concentrations for the rats fed on the experimental diets. Values for Fe in liver ranged from 91.5 to 522.5 µg/g tissue. The three dimensional response surface plot of Fe distribution in liver is shown in Fig. 5. Met(x1) had a significant quadratic effect on liver Fe level (P<0.05). Using stepwise regression, the

most predictive model using the criteria that the last variables entered resulted in a significant improvement for liver Fe was: $y(\text{liver Fe})=144.89+282.32\text{Met}(x1)^2$ (P<0.05). The critical values for the minimum point of liver Fe content were 0.245(g/100g diet) for Met and 0.540(g/100g diet) for Cys.

Values for kidney Fe ranged from 136.5 to 659.0 µg/g tissue and for testis ranged from 71.90 to 136.50 µg/g tissue. In kidney and testis neither Met(x1) nor Cys(x2) had significant effects on Fe levels.

Effects on Zn distribution

The Zn concentrations of liver, kidney and testis are presented in Tab. 6. The values for liver Zn ranged from 115.5 to 212.0 µg/g tissue. Dietary Met and Cys did not have significant response surface effects as far as liver Zn is concerned.

Values for kidney Zn ranged from 97.5 to 146.5 µg/g tissue. Dietary Cys(x2) had a significant effect (P<0.05) on kidney Zn level(Fig.6). Using stepwise regression, the most predictive model using the criteria that the last variable entered resulted in a significant improvement for kidney Zn was: $y(\text{kidney Zn})=107.29+23.12\text{Cys}(x2)$ (P<0.05).

Values for testis Zn ranged from 100.00 to 199.50 µg/g tissue. From the response surface analysis for testis Zn level(Fig.7), dietary Cys(x2) had a significant quadratic effect (P<0.05). Using stepwise regression, the most predictive model using the criteria that the last variable entered resulted in a significant improvement for testis Zn was: $y(\text{testis Zn})=214.44-99.72\text{Cys}(x2)-50.84\text{Met}(x1)^2+63.60\text{Cys}(x2)^2$ (P<0.05).

DISCUSSION

Cd is an ubiquitous environmental pollutant. It is a nonessential and toxic metal and interacts with the metabolism of other essential metals. The addition of certain mineral mixtures in the diet is shown to diminish the toxic effects of Cd (Groten et al. 1991). The differences in toxicity between an unsupplemented Cd diet and some of the Cd diets supplemented with minerals were accompanied by differences in the Cd content of the rat liver and kidneys (Groten et al. 1991). There is accumulating evidence to indicate that there are significant interactions between Cd, Cu, Fe and Zn with respect to their toxicodynamic and toxicokinetic properties (Petering et al. 1971). Cd interferes with absorption of Zn, Fe and Cu. There are several general explanations for the adverse effects of dietary Cd antagonism of essential minerals (Fox 1974). Cd may replace Zn or some metals in an enzyme or at some other site and interfere with a specific metabolic reaction. Cd may displace an essential element or the carrier of an essential element in a transport system, thus disrupting such processes as intestinal absorption, transport and storage within the body and excretion.

Models for the effects of dietary Met and Cys on Cd and Cd-induced changes in distribution of Cu, Fe and Zn in liver, kidney and testis have been determined using response surface regression analysis and three dimensional response surface plotting. Response surface regression analysis has been used as an analytical tool in sensory evaluation (Glovanni 1983; Schutz 1983), in digestibility studies (George et al. 1980), in determination of a balanced diet for maximal body energy gain and body protein gain (Toyomizu et al. 1985) and in evaluating effects of dietary components on lipids (Stewart et al. 1987).

A two-factor central composite rotatable response surface design was used for the

experimental settings for the nine groups of rats studied. With this experimental design, we have attempted for the first time to demonstrate the influence of dietary sulfur containing amino acids such as Met and Cys on Cd distribution and their impact on Cu, Fe and Zn concentrations of liver, kidney and testicular tissues of rats treated with $CdCl_2$ (100ppm) in drinking water daily for a 10 week period. It is a well established fact that the biosynthesis of MT, an unique low molecular weight intracellular protein is induced by a large number of metals including Cd, Cu and Zn. Previous studies have demonstrated that the quantity of MT in a given target organ influences both the toxicodynamic and toxicokinetic profiles of Cd, Zn and Cu(Whanger and Ridlington 1982). The results of our previous studies indicated that Met in the diet had a significant influence on MT levels of liver of rats treated with $CdCl_2$ (100ppm) in drinking water daily for 10 weeks.

The type of dietary protein in purified diets was found to influence markedly the severity of Cd toxicity (Fox et al. 1973). High levels of dietary Cd have led to decreased tissue levels of Cu, Fe and Zn. There is considerable evidence that Cd interferes with absorption of Cu , Fe and Zn. Deficiencies of these elements could lower the threshold for absorption and foster the long-term toxic effects of Cd. It is also possible that at low levels of Cd intake, interactions in processes other than absorption become principally important because of differences in MT formation and biological turnover (Fox 1979). Using a small number of animals in each treatment group with response surface analysis, the data presented in this study indicate that the change of the levels of either Met or Cys in the diet could produce significant effects on the quantity of Cd in the liver, kidney and testicular tissues, Fe in the liver and Zn in the kidney and testis of the rats treated with $CdCl_2$ (100ppm) in drinking water daily for 10 weeks. Changes such as a decrease in Cd burden in vital target organs such as

liver, kidney and testis at higher intake of dietary Met and Cys could be beneficial in Cd-induced toxicity.

Fe was the most effective mineral in rats for preventing Cd accumulation (Fox et al. 1980). Previous studies have indicated a negative correlation between the Fe and Cd content in the diet and the Cd and Fe absorption in animals. It is reported that the uptake of Cd in the intestinal mucosa and its transport from the intestine to other compartments of the body is increased in animals with Fe deficiency (Flanagan et al. 1978). The uptake and transport of Cd was found to be lowered in quail fed on diets with Fe supplementation (Fox et al. 1980). Cd competes with the Fe transfer system mainly by binding to mucosal transferrin. Mucosal transferrin appears to be an important determinant in Fe uptake in the intestine (Huebers et al. 1983). Injection of Fe greatly increased hepatic MT production in rats (Petro and Hill 1987). Changes in the MT level in the intestine by Fe may result in an alteration of Cd retention (Eaton and Toal 1982). It has been shown that dietary Met had a significant increase in liver Fe in our experiments. This is likely related to increased Fe absorption. Changes in the MT level in the intestine by sulfur containing amino acids might have resulted in an alteration on Fe absorption.

The beneficial effect of supplementation of Zn together with Cu has been reported by Jacobs et al. (1978). Zn caused the protective effect against Cd accumulation (Jacobs et al. 1983). Cu and Zn are essential metals, while Cd has no recognized biological role. These three heavy metals bind preferentially to the same protein, albumin, in the bloodstream (Suzuki et al. 1989). and accumulate in the liver bound to similar proteins, including MT (Kagi and Kojima 1987). Cd competes with Zn for binding sites on MT, which is important in the storage and transport of Zn during development. Excess Zn is known to protect against

the Cd-induced injury to the testis (Gunn et al. 1961). It has been recognized that only small amounts of Cd are accumulated by the testis, and this accumulation does not lead to displacement of Zn. On the contrary, the Zn content of both the rat and mouse testis remains unchanged for a short time after the parenteral injection of Cd, and then increases progressively. This is possibly associated with the proliferation of interstitial and, particularly, connective tissue. In both the liver and kidney, the Zn content is not depressed but increases with the uptake of Cd (Webb 1971). In the present study, our results indicate that dietary Cys has a significant effect on testis Cu. This is possibly related to an increase in MT synthesis and increased Cu absorption. Dietary Cys significantly increased both kidney and testicular tissue Zn levels. Because Cd interferes with the absorption and metabolism of Zn, dietary sulfur containing amino acids might have changed these processes. Additional experiments will be necessary in optimizing dietary levels of either Met or Cys to minimize the effects of Cd burden on liver, kidney and testicular tissues.

In summary, the results of this present study indicate that dietary Met and Cys alone or in combination could reduce Cd burden in liver, kidney and testis and affect liver Fe, testis Cu and kidney and testicular tissue Zn concentrations. Because of the complex toxicokinetic and toxicodynamic interactions between Cd and other essential metals, it would be very difficult to selectively alter one of these metals in a consistent manner in a given target organ. Further studies will be necessary to clearly demonstrate dietary levels of either Met or Cys to minimize Cd burden without any drastic changes in the concentrations of other essential trace minerals in vital organs.

REFERENCE

Eaton DL and Toal BF (1982) Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. Toxicol Appl Pharm 66:134-142

Fielder RJ, Dale EA (1983) Cadmium and its compounds, toxicity review 7, health and safety executive, Crown Copyright, London, p88

Flanagan PR, McLellan J and Haist J (1978) Increased dietary cadmium absorption in mice and human subjects with iron deficiency. Gastroenterology 74:841-846

Flick DF, Kraybill HF and Dimitroff JM (1971) Toxic effects of cadmium: A review. Environ Res 4:71-85

Fox MRS (1974) Effects of essential minerals on cadmium toxicity. J Food Sci 39:321-324

Fox MRS (1979) Nutritional influences on metal toxicity: cadmium as a model toxic element. Environ Health Perspet 29:95-104

Fox MRS, Jacobs RM and Jones AO (1979) Effects of nutritional factors on metabolism of dietary cadmium at levels similar to those of man. Eviron Health Perspect 28:107-114

Fox MRS, Jacobs RM, Jones AOL, Fry BE and Stone CL (1980) Effects of vitamin C and iron on cadmium metabolism. Annal New York Aced Sci 355:249-261

Friberg L, Piscator M, Nordberg GE, Kjellstrom T (1974) Cadmium in the environment 2nd ed. CRC Press, Cleveland, U.S.A. pp23-201

Friberg L, Piscator M, Nordberg GF and Kjellstrom T (1976) Cadmium in the environment 2nd ed. CRC Press, Cleveland, U.S.A. pp-21

George JR, Harbers LH and Reeves RD (1980) Digestive responses of rats to fiber type, level and particle size. Nutr Rep Int 21:312-322

Glovanni M (1983) Response surface methodology and product optimization. Food Tech 37:41-45

Groten JP, Sinkeldam EJ, Muys T, Luten JB and Bladeren PJ (1991) Interaction of dietary Ca, P, Mg, Mn, Cu, Fe, Zn and Se with the accumulation and oral toxicity of cadmium in rats. J Chem Toxic 29:249-258

Gunn SA, Gould TC and Anderson WAD (1961) Zinc protection against cadmium injury to rat testis. Arch Path 71:987-991

Gunn SA, Gould TC and Anderson WAD (1966) Protective effect of thiol compounds

against cadmium-induced vascular damage to testis. Proc Soc Exp Biol Med 122:1036-1039

Huebers HA, Hubers E, Csiba E, Rummel W and Finch CA (1983) The significance of transferrin for intestinal iron absorption. Blood 61:283-290

Jacobs RM, Jones AOL, Fox MRS and Fry BE (1978) Retention of dietary cadmium and the ameliorative effect of zinc, copper, and manganese. J Nutr 108:22-32

Jacobs RM, Jones AOL, Fox MRS and Lener J (1983) Effects of dietary zinc, manganese and copper on tissue accumulation of cadmium by Japanese quail. Proc Soci Experi Bio Med 172:34-38

Jorhem L (1993) Determination of metals in foodstuffs by atomic absorption spectrophotometry after dry ashing. J AOAC Intern 76:798-813

Kagi JH and Kojima Y (1987) Chemistry and biochemistry of metallothionein. (Kagi JH and Kojima Y Eds.) Birkhauser/Verlag Basel. pp. 25-61

Mills CF and Delgarnd AC (1972) Copper and zinc status of ewes and lambs receiving increased dietary concentration of cadmium. Nature 239;171-173

Nriagu JO (1980) Cadmium in the environment part II health effects. John & Sons, New York, pp 839

Petering DG, Johnson MA and Stemmer K (1971) Studies of zinc metabolism in the rat. Arch Environ Health 23:93-96

Petering HG, Choudhury H and Stemmer KL (1979) Some effects of oral ingestion of cadmium on zinc, copper, and iron metabolism. Environ Health Perspect 28:97-106

Petro A and Hill CH (1987) Response of hepatic metallothionein to iron administration. Bio Trace Ele Res 14:255-263

Samarawicrama GP (1979) The chemistry, biochemistry and biology of cadmium: Biological effects of cadmium in mammals (Webb M ed.). Elsevier/North-Holland Biomedical Press, New york. pp. 341-421

Schutz HG (1983) Multiple regression approach to optimization. Food Tech 37:46-62

Stewart JR, Fryer EB and Fryer HC (1987) Effects of dietary fiber, carbohydrate, lipid and protein levels on serum and liver lipids in rats. J Nutr 117:650-659

Suzuki KT, Karasawa A and Yamanaka K (1989) Binding of copper to albumin and participation of cysteine in vivo and in vitro. Arch Biochem Biophys 273:572-577

Toyomizu M, Akiba Y, Matsumoto T and Horiguchi M (1985) Response surfaces analysis

of body protein and energy gains in growing chicks fed diets over the entire range of compositions of protein, fat and carbohydrate. J Nutr 115:61-69

Waalkes MP, Kovatch R and Rehm S (1991) Effect of chronic dietary Zn deficiency on cadmium toxicity and carcinogenesis in the male Wistar rat. Toxicol Appl Pharmacol 108:448-456

Webb M (1971) Biochemical effects of Cd-injury in the rat and mouse testis. J Reprod Fert 30:83-98

Whanger PD and Ridlington JW (1982) Role of metallothionein in zinc metabolism, in: Biological role of metallothionein. (Foulkes EC ed.), Elsevier, Amsterdam. pp. 263-277

TABLE 3

Cd concentrations(Mean ±SE) of kidney, liver and testicular tissues of rats fed on the experimental diets containing variable levels of Met and Cys with CdCl (100ppm) in drinking water for 10 weeks

Diet no.	% of diet		Cd(µg/g)		
	Met	Cys	kidney	liver	testis
01	0.22	0.12	95.33± 2.49	97.17± 2.46	1.79±0.25
02	1.28	0.12	123.83± 6.49	130.00± 7.56	2.81±0.18
03	0.22	0.68	80.33± 3.57	54.33± 1.25	1.35±0.39
04	1.28	0.68	93.00± 5.00	68.25± 2.75	2.00±0.01
05	0.00	0.40	88.50± 4.30	50.17± 7.76	1.29±0.08
06	1.50	0.40	93.00±11.34	67.00±17.45	1.57±0.46
07	0.75	0.00	98.17±18.60	68.33±32.56	1.69±0.41
08	0.75	0.80	59.67± 0.94	37.83± 4.09	0.92±0.55
09	0.75	0.40	83.07± 5.83	66.27±10.31	1.31±0.30

TABLE 4

GSH contents (Mean ±SE) of brain, heart, liver and testicular
tissues of rats fed on the experimental diets containing variable levels
of Met and Cys with CdCl$_2$ (100ppm) in drinking water for 10 weeks

| diet no. | % of diet | | GSH(µg/100mg) | | | |
	Met	Cys	brain	heart	liver	testis
01	0.22	0.12	87.54±20.08	210.68± 6.10	582.32± 39.95	234.36± 4.11
02	1.28	0.12	106.83±22.24	216.17± 3.68	634.76± 44.08	188.55±10.61
03	0.22	0.68	65.82±14.23	218.19±12.26	545.56± 21.18	184.42±27.53
04	1.28	0.68	131.41± 4.63	204.27± 9.19	366.10± 31.20	179.10± 9.59
05	0.00	0.40	89.93± 5.01	214.89± 8.14	521.73± 60.05	219.34±20.98
06	1.50	0.40	109.99±10.59	220.57± 1.55	599.05± 56.11	237.71± 5.33
07	0.75	0.00	84.99±14.30	205.77±15.92	494.80± 50.48	324.48±26.97
08	0.75	0.80	112.33±13.15	223.02± 8.47	696.45± 46.04	300.94±19.13
09	0.75	0.40	98.72± 3.43	210.22± 7.09	620.67± 74.28	324.67±20.30

TABLE 5

MDA levels(Mean ±SE) of brain, liver and testicular tissues
of rats fed on the experimental diets containing variable levels
of Met and Cys with CdCl (100ppm) in drinking water for 10 weeks

	% of diet		MDA(nmol/g)		
Diet no.	Met	Cys	brain	liver	testis
01	0.22	0.12	143.97± 3.43	188.77±21.60	119.03±2.88
02	1.28	0.12	127.62±10.32	195.45± 1.43	121.83±7.36
03	0.22	0.68	121.90± 5.23	217.74±13.58	119.96±5.72
04	1.28	0.68	149.05± 0.75	211.97± 0.49	119.49±5.87
05	0.00	0.40	145.65± 1.75	185.50± 6.83	120.89±0.66
06	1.50	0.40	137.12± 4.10	212.32± 5.18	126.97±3.03
07	0.75	0.00	138.05± 1.15	226.32± 8.75	122.76±4.12
08	0.75	0.80	140.92± 3.06	255.75±10.48	113.89±6.30
09	0.75	0.40	137.45± 2.26	228.22± 11.80	112.47±6.08

TABLE 4

GSH contents (Mean ±SE) of brain, heart, liver and testicular
tissues of rats fed on the experimental diets containing variable levels
of Met and Cys with $CdCl_2$ (100ppm) in drinking water for 10 weeks

diet no.	% of diet		GSH(µg/100mg)			
	Met	Cys	brain	heart	liver	testis
01	0.22	0.12	87.54±20.08	210.68± 6.10	582.32± 39.95	234.36± 4.11
02	1.28	0.12	106.83±22.24	216.17± 3.68	634.76± 44.08	188.55±10.61
03	0.22	0.68	65.82±14.23	218.19±12.26	545.56± 21.18	184.42±27.53
04	1.28	0.68	131.41± 4.63	204.27± 9.19	366.10± 31.20	179.10± 9.59
05	0.00	0.40	89.93± 5.01	214.89± 8.14	521.73± 60.05	219.34±20.98
06	1.50	0.40	109.99±10.59	220.57± 1.55	599.05± 56.11	237.71± 5.33
07	0.75	0.00	84.99±14.30	205.77±15.92	494.80± 50.48	324.48±26.97
08	0.75	0.80	112.33±13.15	223.02± 8.47	696.45± 46.04	300.94±19.13
09	0.75	0.40	98.72± 3.43	210.22± 7.09	620.67± 74.28	324.67±20.30

FIGURE LEGENDS

Fig. 1: Three-dimensional plot of liver Cd (μg/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. $y = 79.03 - 46.28x1 + 11.44x2 + 10.61x1^2 + 13.31x2^2 - 39.63x1x2$.

Fig. 2: Three-dimensional plot of kidney Cd (μg/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. $y = 99.18 - 29.05x1 - 12.38x2 + 10.84x1^2 + 24.32x2^2 - 35.23x1x2$.

Fig. 3: Three-dimensional plot of testis Cd (μg/g tissue) as a function of percent of Mex(x1) with percent of Cys(x2) in the diet. $y = 1.74 - 0.94x1 - 0.17x2 + 0.43x1^2 + 0.67x2^2 - 1.02x1x2$.

Fig. 4: Three-dimensional plot of testis Cu (μg/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. $y = 13.09 + 2.39x1 - 5.85x2 - 3.91x1^2 + 4.43x2^2 - 1.18x1x2$.

Fig. 5: Three-dimensional plot of liver Fe (μg/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. $y = 205.28 - 330.35x1 - 73.55x2 + 628.31x1^2 + 58.67x2^2 + 41.36x1x2$.

Fig. 6: Three-dimensional plot of kidney Zn (μg/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. $y = 112.55 - 15.77x1 + 18.93x2 + 5.80x1^2 + 0.46x2^2 + 8.79x1x2$.

Fig. 7: Three-dimensional plot of testis Zn (μg/g tissue) as a function of percent of Met(x1) with percent of Cys(x2) in the diet. $y = 202.83 + 43.56x1 - 88.41x2 - 80.68x1^2 + 62.17x2^2 - 23.78x1x2$.
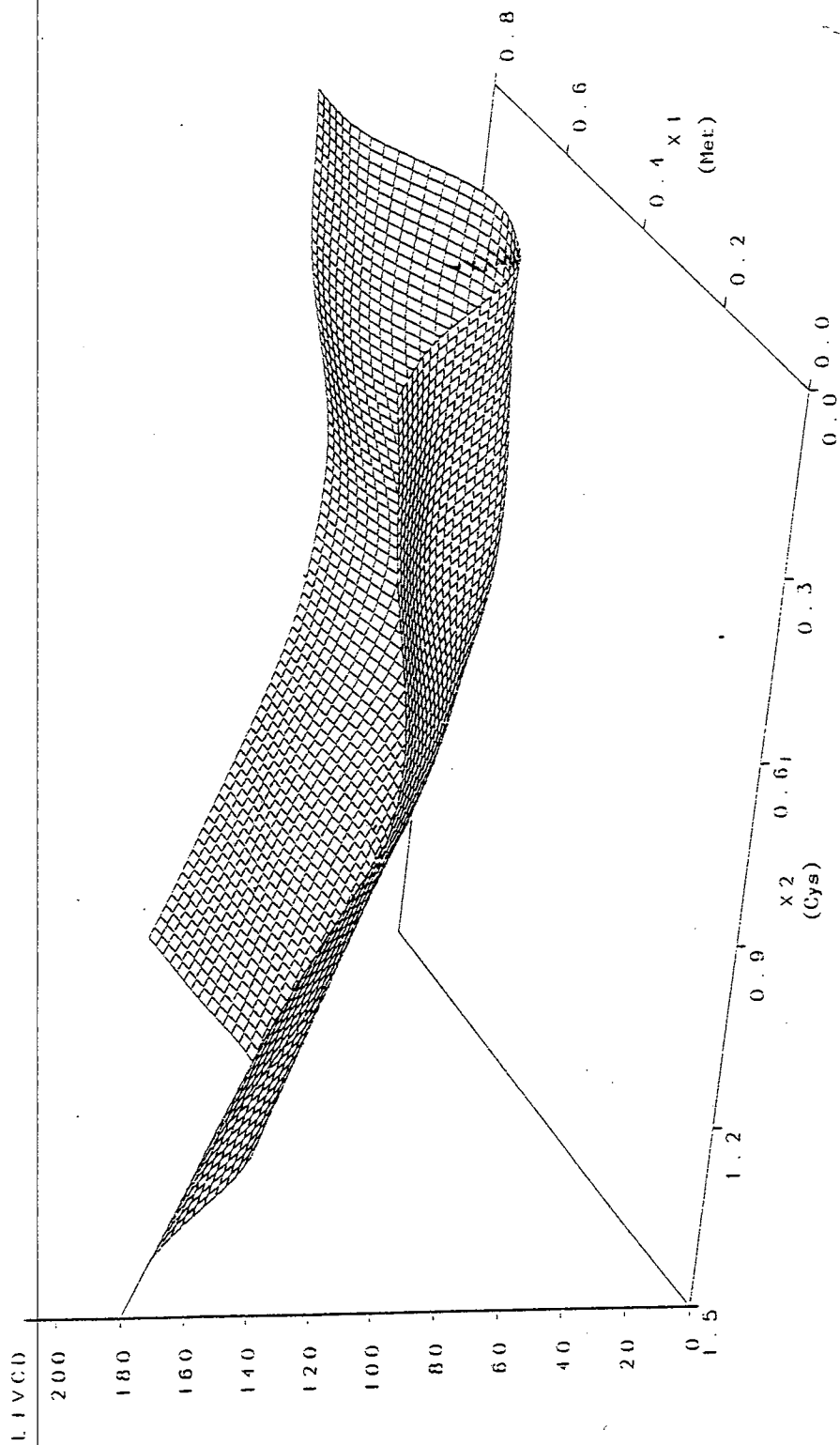
Fig. 1

Fig. 2
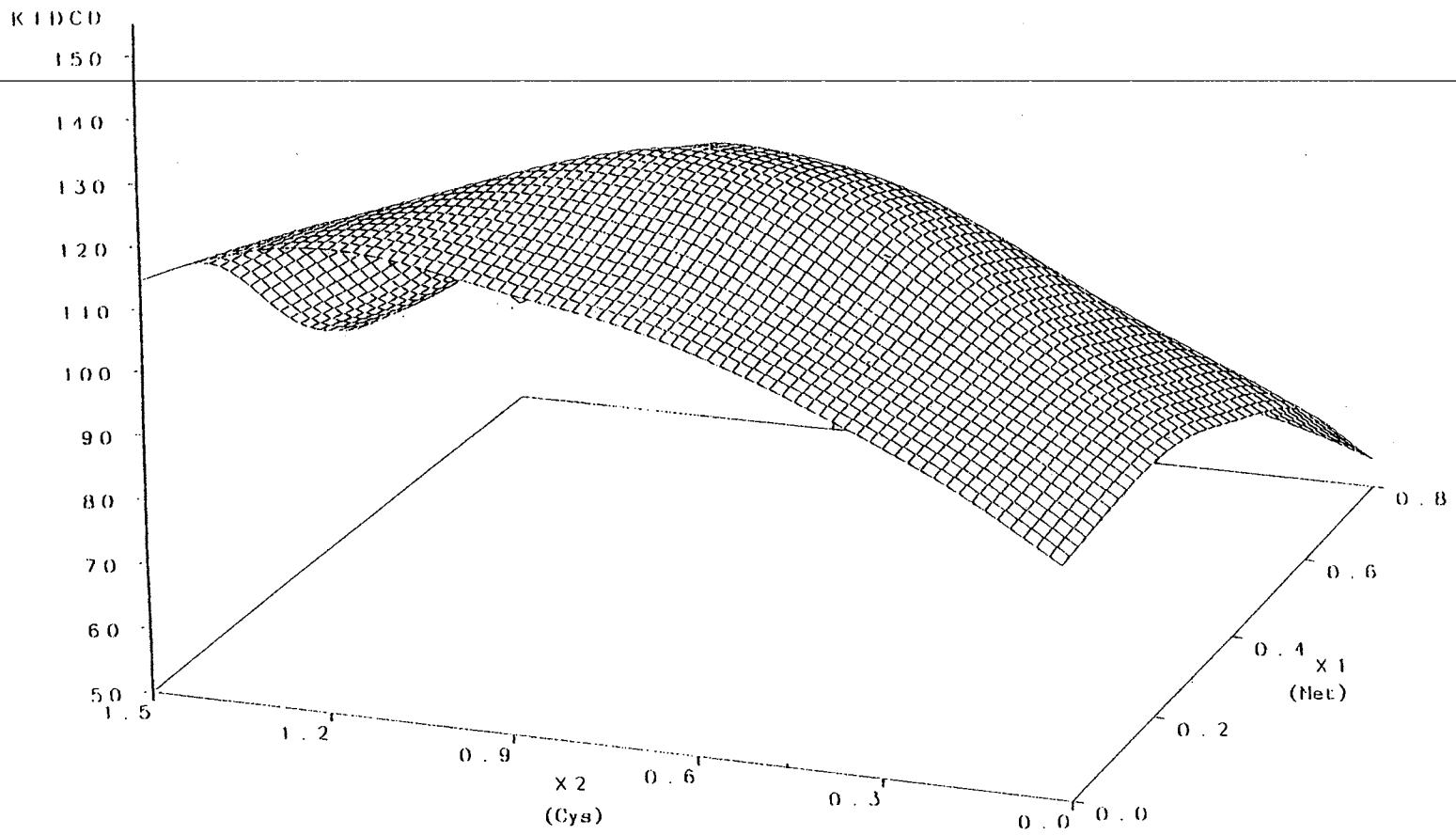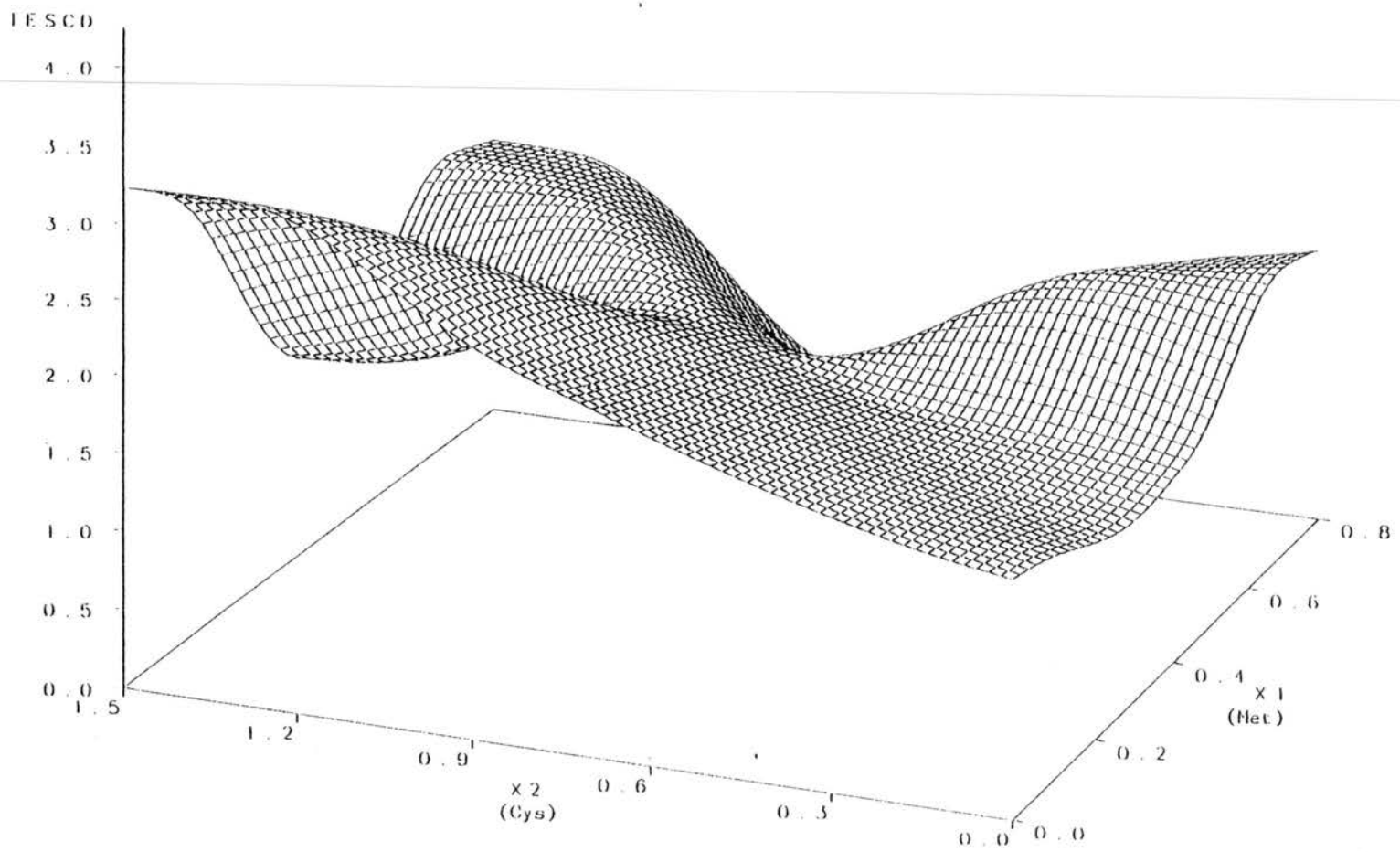
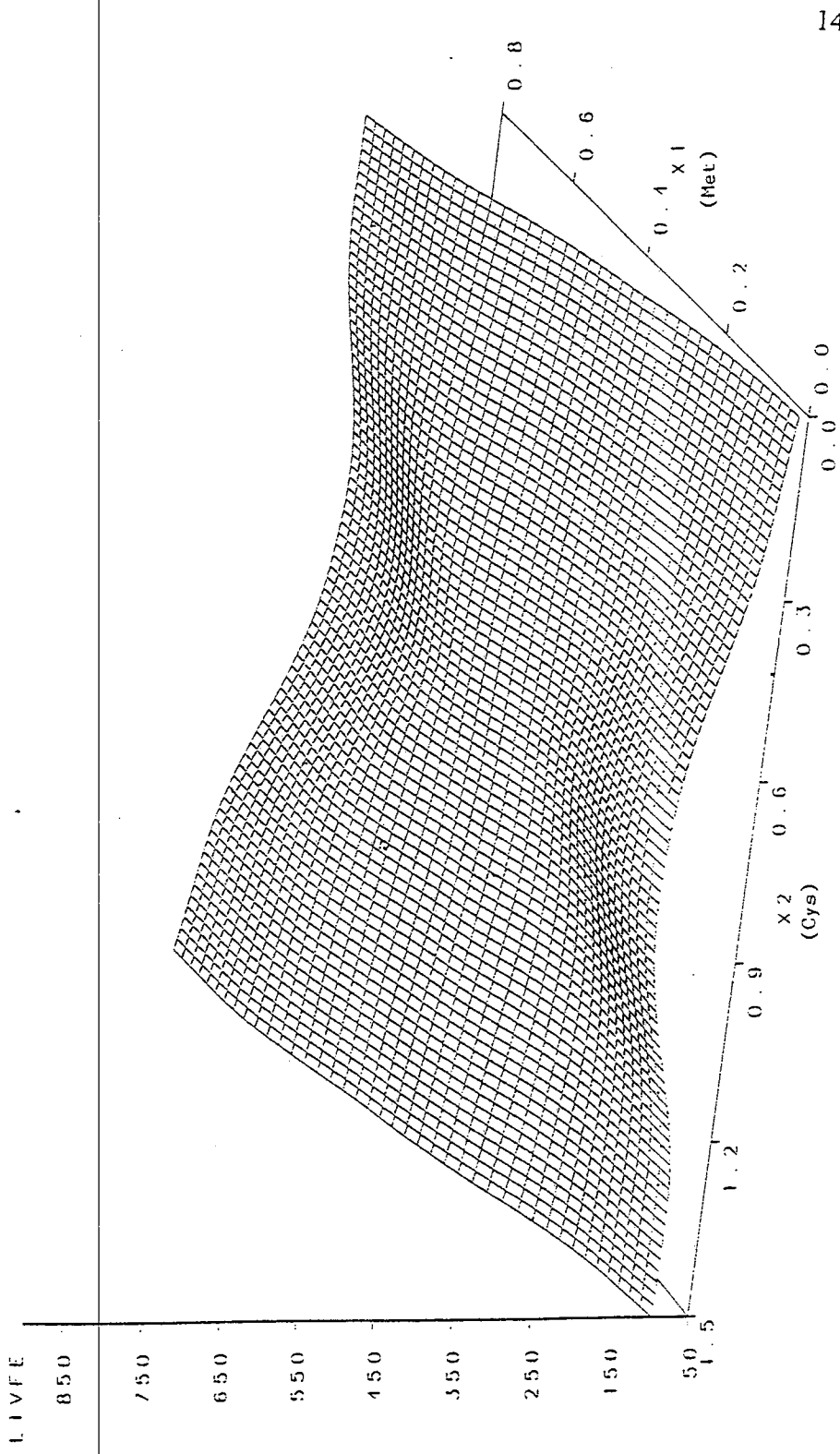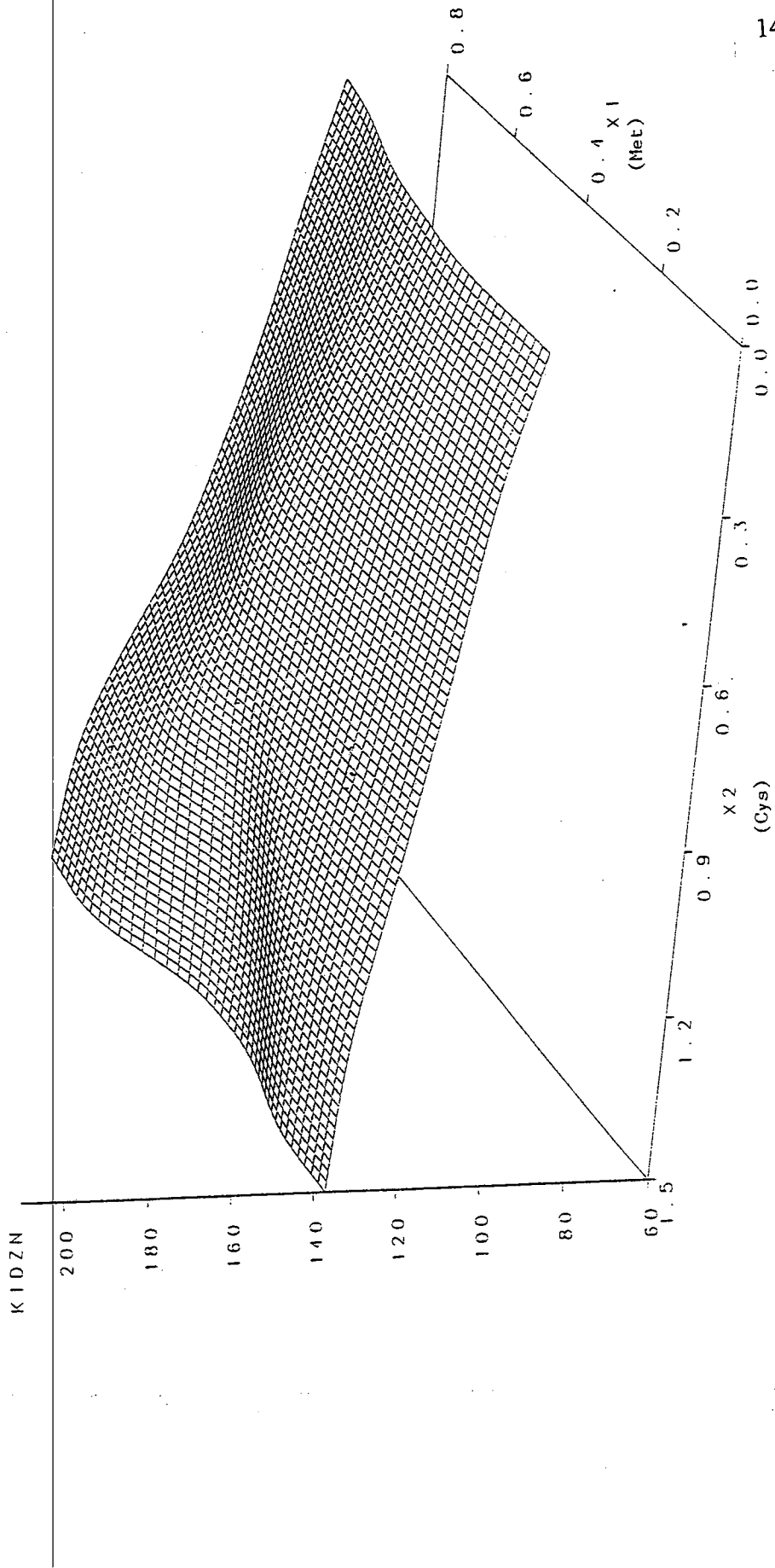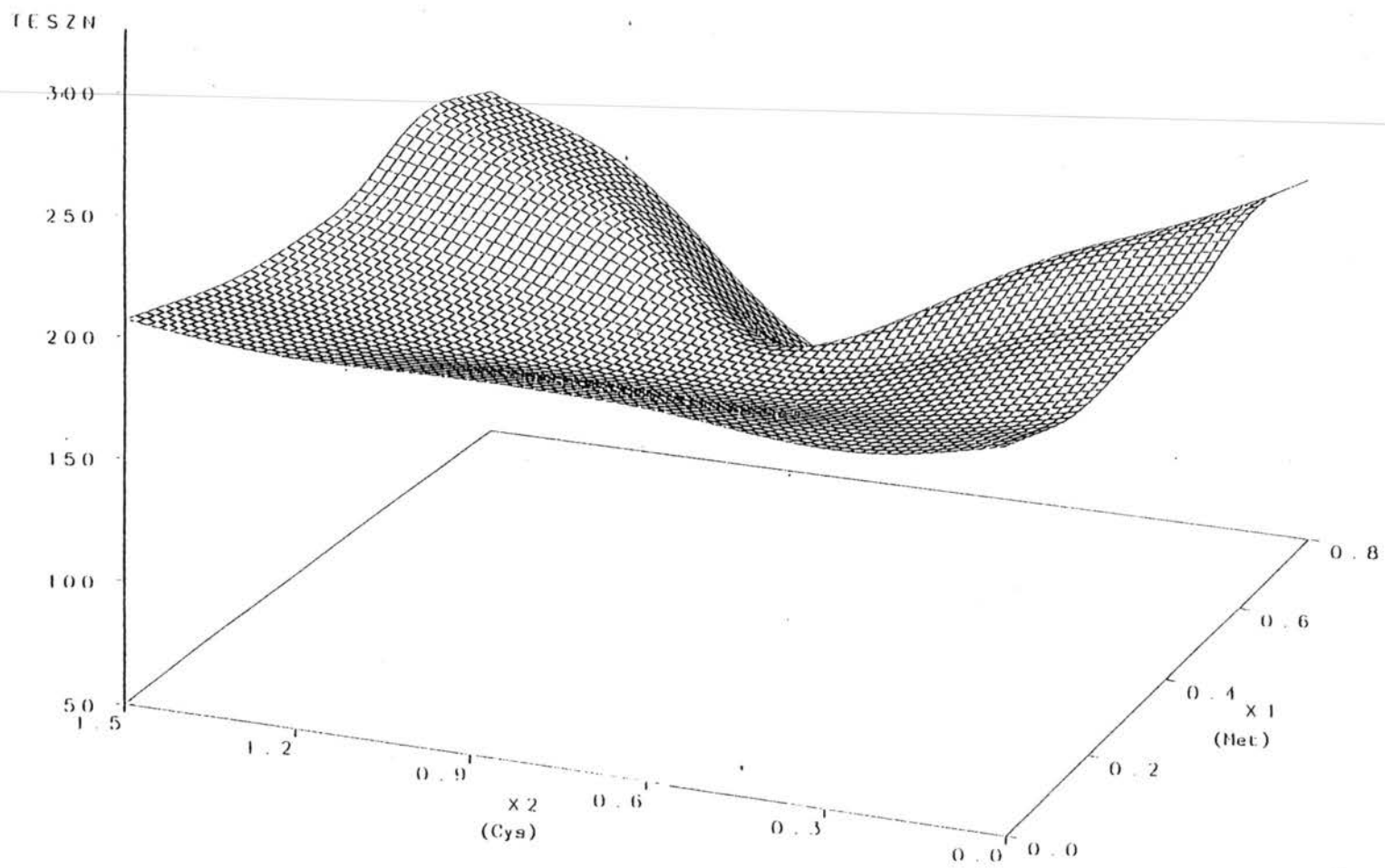Fig. 3

Fig. 4

Fig. 5

Fig. 6

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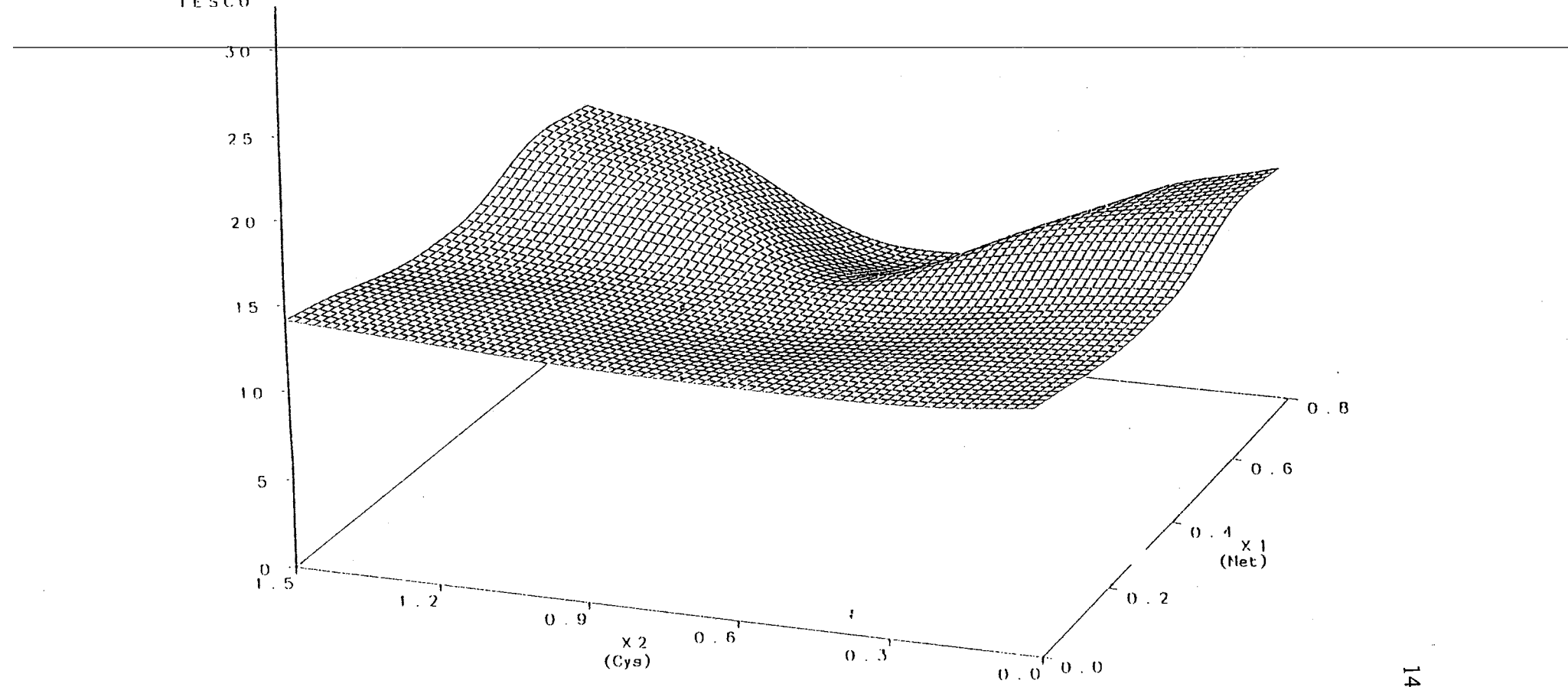
Fig. 7

CHAPTER VI

SUMMARY AND CONCLUSIONS

Cd is a relatively rare element and commercial Cd production started at the beginning of this century. Cd is released to the air, land and water by human activities. Occupational and environmental pollution are the main sources of Cd exposure. Cd possesses a very long biological half-life and acts as a cumulative poison in human and higher animals. Pulmonary absorption is higher than gastrointestinal absorption. Gastrointestinal absorption of Cd is influenced by the type of diet and nutritional status. Cd absorbed from the lungs and the gastrointestinal tract is mainly stored in the liver and kidneys, where more than half of the body burden will be deposited. The toxic effects of exposure to Cd include anemia, dermatitis, testicular degeneration or atrophy, reduced growth rate, liver and kidney damage, cardiovascular disorders, pulmonary edema and emphysema, teratogenic malformations and increased mortality.

Testis is one of the most sensitive tissues to the acute toxic and chronic carcinogenic effects of Cd. Cd exerts its toxic effects on testicular vascular endothelium which could lead to ischemia, hypoxia and lipid peroxidation followed by generation of highly reactive hydroxyl free radicals in the testicular tissues. To determine the relationship between Cd induced testicular toxicity and hydroxyl free radical generation, male, CD-1 mice were given $CdCl_2$ and salicylate was used to trap hydroxyl free radicals in the testis. Two main products are 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) formed by

hydroxyl addition to salicylate. The results indicated that the concentrations of both 2,3-DHBA and 2,5-DHBA, determined with high performance liquid chromatography with electrochemical detector(HPLC-EC) in the testes of Cd-treated mice were significantly higher than those of mice without the treatment of Cd. This study provides direct evidence for the involvement of hydroxyl free radicals in Cd-induced testicular toxicity in mice. It provides an important information for further study of the mechanism of testicular toxicity caused by Cd.

Cd-induced biochemical changes were characterized in male, CD-1 mice testes. ATPase, an integral part of the cell membrane plays an important role in the active transport of Na^+ and K^+ across cell membrane. Cd inhibited the testes microsomal Na^+, K^+-ATPase activity *in vitro* and *in vivo*. The inhibitory concentration was 30-150μM and the concentration for half maximal inhibition (IC_{50} value) was 90μM over 5 minutes preincubation. Cd treatment for 2 days significantly inhibited testis Na^+, K^+-ATPase *in vivo*.

GSH is a nonprotein thiol which is involved in cellular protective mechanisms that attenuate responses to toxic agents. The amount of testicular GSH and the ratio of GSH/GSSG decreased in Cd treated mice.

Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids, and the eventual destruction of membrane lipid. Cd increased the testicular malondialdehyde (MDA) level due to lipid peroxidation both *in vitro* and *in vivo*.

Vitamin E is involved in the overall cellular anti-oxidant defense against deleterious effects of reactive oxygen species. Our results indicated that pretreatment with vitamin E

produced a significant reduction in the Cd induced increase in the formation of both 2,3-DHBA and 2,5-DHBA as indices of hydroxyl free radicals in mice testes. This finding provided direct evidence that vitamin E could be effective in partially preventing Cd-induced toxicity via the reduction in hydroxyl free radical formation and lipid peroxidation in testicular tissues.

The nutritional status viz. dietary proteins and essential trace elements greatly influence the metabolic fate and toxicity of Cd. Low protein diets enhance Cd toxicity while high protein diets reduce its toxicity. The accumulation and retention of Cd are also influenced by dietary compositions. GSH and MT present in cells of the target organs have been considered to play a critical role in detoxification of Cd. The structure of GSH is r-GLU-CYS-GLY. Mammalian MT contains 61 amino acids, of which 20 are cysteine. GSH and MT are two major intracellular cysteine pools in liver. Sulfur containing amino acids such as cysteine, cystine and methionine also play an important role in Cd toxicity. Cysteine can reverse the toxic effects of Cd. Other sulfur amino acids are related to cysteine metabolism. Cystine and methionine occur normally in our food intakes. Cysteine and cystine are in fact easily interconvertible in the body.

A central composite rotatable design with two x-variables was used in the experimental design and response surface regression analysis was used as an analytical tool to determine the influence of dietary sulfur containing amino acids such as cystine(Cys) and methionine(Met) either alone or in combination on Cd-induced biochemical changes and on distribution of Cd , Cu, Fe and Zn in target organs of male, Sprague-Dawley rats. The regression coefficients and estimated parameters of these response surfaces and three dimensional plots were developed for each of biochemical markers and each of the metal

distribution. The optimal stepwise regression equation was selected for each variable, using the criteria that the last variable entered resulted in a significant improvement in the regression equation for each of the biochemical markers and tissue trace metals. In the study, it has been shown that MT level in the liver was increased as the dietary Met increased at a higher Cys intake. This is likely to be related to MT synthesis. The dietary Met and Cys produced a significant increase in the brain and testis GSH levels signifying the importance of sulfur containing amino acids in the *in vivo* biosynthesis of GSH. Modification of diets with various levels of Met and Cys may not be a reasonable approach to reduce or prevent Cd-induced lipid peroxidation of vital organs and to increase intestinal total ATPase and Na^+, K^+-ATPase activities. However, Met at a higher level produced a significant increase in Mg^{2+}-ATPase activity in the intestinal mucosa.

Additionally, increased levels of Met and Cys in the diet produced a significant reduction in the liver, kidney and testicular tissues Cd burden. The significance of these results are discussed in relation to Cd-induced biochemical changes and their impact on Cd, Cu, Fe and Zn levels in liver, kidney and testicular tissues. Further studies will be necessary in optimizing the levels of Met and Cys either alone or in combination in the diet which would minimize Cd-induced toxic effects via an increase or decrease in the appropriate target organ biochemical changes induced by Cd.

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